SEED GERMINATION AND MEDICINAL PROPERTIES OF *Alepidea* SPECIES

By

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In the

Research Centre for Plant Growth and Development
School of Biological and Conservation Sciences
University of KwaZulu-Natal
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STUDENT DECLARATION

Seed Germination and Medicinal Properties of *Alepidea* Species

I, ROFHIWA BRIDGEHT MULAUDZI, Student Number 207527112

declare that :

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We hereby declare that we acted as Supervisors for this MSc student:

Student's Full Name: ROFHIWA BRIDGEHT MULAUDZI

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Thesis Title: SEED GERMINATION AND MEDICINAL PROPERTIES OF *ALEPIDEA* SPECIES

Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed Examiners.

SUPERVISOR: _____ PROFESSOR J VAN STADEN

CO-SUPERVISOR: _____ DR JF FINNIE

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"Without perseverance and courage, how would a child learn to walk?" Mary Nichols.

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"In the end we will remember not the words of our enemies, but the silence of our friends". Martin Luther King jr.

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ABSTRACT

The rhizomes of *Alepidea amatymbica* and *Alepidea natalensis* are used for medicinal purposes. Because of the increase in demand for these plants the species is becoming scarce. As the seed biology of neither species is well defined, conditions as well as treatments required for optimum germination and vigour were studied. Seeds were exposed to various physical factors such as varying light and temperature conditions and cold stratification, sowing depth and seed storage. The effects of smoke-water, butenolide (3-methyl-*2H*-furo [2, 3-*c*] pyran-2-one) a novel smoke compound and chemical substances (gibberellins, kinetin and KNO_3) were also tested in order to improve seed germination.

Alepidea amatymbica and *A. natalensis* achieved the highest seed germination (72.5% and 80%, respectively) at 25 ℃ under a 16 h photoperiod with a mean germination time (MGT) of 18 and 12 days, respectively. Phytochrome studies showed that *A. natalensis* requires light for germination. Cold stratification (5 ℃) for 14-28 days significantly improved the percentage germination of both species (> 90%) compared to non-stratified seeds (control) at 25 ℃ under a 16 h photoperiod. Sowing *A. amatymbica* and *A. natalensis* seeds at a depth of 0.5 cm resulted in higher percentage germination compared to 2.5 cm. The highest emergence rate for *A. amatymbica* was 40% at a sowing depth of 0.5 cm and the lowest emergence rate was 3% at 2.5 cm. Six months storage of *A. natalensis* seeds at room temperature (25 ± 2 ℃) showed maximum germination (99%) with a MGT of 9 days. Smoke-water treatment of *A. amatymbica* seeds significantly enhanced germination from 72% to 91%. Smoke and butenolide at 10 ℃ and 25 ℃ promoted germination of *A. natalensis* seeds in a 16 h photoperiod. Smoke-water application significantly improved both germination and seedling vigour of *A. natalensis*. GA_3 (10^{-8} M) was the best treatment for achieving maximum percentage germination of *A. natalensis* seeds.

Antibacterial (two Gram-positive bacteria: *Bacillus subtilis*, *Staphylococcus aureus* and two Gram-negative bacteria: *Escherichia coli*, *Klebsiella pneumoniae*), antifungal (*Candida albicans*), anti-inflammatory (COX-1 and -2) and genotoxicity tests (Ames test) were carried out on petroleum ether (PE), dichloromethane

(DCM), 80% ethanol (EtOH) and water extracts of the two *Alepidea* species. Water extracts of *A. natalensis* rhizomes exhibited high activity (MIC values of 0.78 mg/ml) against the four bacterial strains. High activity was also observed in the PE and DCM leaf extracts of the same plant against the Gram-positive bacteria. The PE and DCM extracts of *A. amatymbica* rhizomes exhibited the best activity (MIC values of 0.39 mg/ml) against *Bacillus subtilis*. The rest of the extracts showed low activity (MIC values >1 mg/ml). All the extracts showed activity against *Candida albicans*, with *A. natalensis* leaf extracts exhibiting the highest antifungal activity with MIC values of 0.88, 0.20 and 0.78 mg/ml for PE, DCM and EtOH, respectively. EtOH extracts had inhibition less than 40% for both *A. natalensis* and *A. amatymbica*. All the PE extracts showed higher inhibitory activity for COX-2 than for COX-1. PE and DCM extracts had percentage inhibitions above 70% in both COX-1 and COX-2 assays. The Ames test for genotoxicity revealed that none of the plant extracts were genotoxic to the *Salmonella* TA98 tester strain.

PUBLICATIONS FROM THIS THESIS

1. **R.B. Mulaudzi**, A.R. Ndhlala, J.F. Finnie and J. Van Staden, (2009). Antimicrobial, anti-inflammatory and genotoxicity tests of *Alepidea amatymbica* and *Alepidea natalensis.* Accepted for publication in *South African Journal of Botany*.

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4NQO	4-Nitroquinoline-1-oxide
AmpB	Amphotericin B
ANOVA	One-Way Analysis of Variance
ATCC	American Type Culture Collection
COX	Cyclooxygenase
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
EtOH	Ethanol
GA_3	Gibberellic acid
INT	*p*-Iodonitrotetrazolium chloride
MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
MGT	Mean germination time
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
MPD	Morphophysiological dormancy
NCCLS	National Committee of Clinical Laboratory standards
NSAIDs	Non-steroidal anti-inflammatory drugs
PE	Petroleum ether
PGs	Prostaglandins
PPF	Photosynthetic photon flux density
SVI	Seedling vigour indices
TTC	2,3,5-Triphenyl tetrazolium chloride
USD	United States Dollar
YM	Yeast malt

CHAPTER 1. INTRODUCTION

1.1. Plants in medicine

Medicinal plants have always played a major role in man's life. There is a close relationship between plants and human beings. Man depends on plants for his existence and this relationship must be sustainable. Unsustainable utilization of medicinal plants could lead to their extinction (AMPONSAH *et al.,* 2002).

The sustainable use of medicinal plants has become an important issue globally. In South Africa, there is an increase in the demand for medicinal plants because of population growth. Many medicinal plants are being exploited in their natural habitats, threatening future supplies (KULKARNI *et al.,* 2005b).

The demand for medicinal plants is already too high and meeting the demand by sustainable means will be extremely difficult (VAN STADEN, 1999). The increasing demand has resulted in unprecedented growth in commercial harvesting and the sale of traditional medicines (CUNNINGHAM, 1988). The commercial trade is associated with heavy exploitation of some medicinal plants. *Siphonochilus natalensis* is reported to have disappeared from known localities in the Natal province in 1900 (MEDLEY-WOOD and FRANKS, 1911).

Heavy exploitation of the indigenous species is mainly for commercial trade (CUNNINGHAM, 1988). According to AFOLAYAN and ADEBOLA (2004), the collection of medicinal plants has become a form of rural self-employment in Africa which is generating income to the poor.

1.2. Traditional medicine

Traditional medicine is defined as health practices, approaches, knowledge and beliefs, incorporating plants, animals and mineral based medicines as well as spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being (WHO, 2001).

In Africa, Asia and Latin America people use traditional medicine to meet some of their primary health care needs. Most of the African population (80%) uses traditional medicine (SPARG *et al*., 2002). According to SPARG (2003), modern health care cannot meet the needs of the entire world's population. There is a need to promote traditional medicine as a means to improve the health in many developing countries (LE GRAND and WONDERGEM, 1989). For this reason, medicinal plants have become the focus of intense studies in recent years to determine whether their traditional uses are supported by actual pharmacological effects or whether they are merely based on folklore (SPARG *et al*., 2002). Many of the plants that are used in traditional medicine are now known to contain pharmacologically active compounds.

1.3. Medicinal plants in South Africa

South Africa has a rich plant biodiversity, with many of the plants having medicinal uses. Such a rich biodiversity could be easily lost due to overexploitation (AFOLAYAN and ADEBOLA, 2004). It is estimated that over half a million people are directly involved in the medicinal plant trade in South Africa. Studies of the trade in medicinal plants in the Eastern Cape Province revealed a minimum of 166 plant species providing 525 tonnes of plant material valued at USD 3.4 million annually (DOLD and COCKS, 2002). From the species documented, 93% are being harvested unsustainably and 34% of the species have been prioritised for conservation management. In KwaZulu-Natal, about 6 million people are known to be using indigenous medicinal plants (MANDER, 1998). More than 4000 tonnes of plant material are traded annually at a value of USD 7.6 million a year which is

equivalent to one third of the annual maize harvest for the province (MANDER, 1998). According to MANDER and LE BRETON (2005) there are up to 100 million traditional-remedy consumers in southern Africa and about 500,000 traditional healers. Most households in South Africa spend between 4 to 6% of their annual income on indigenous medicine and services (MANDER, 1998). However, the increase in agriculture, forestry, industrial, urban housing and development of private and communal land has impacted negatively on the environment which could results in extinction of many popular medicinal plants (MANDER *et al*., 1996).

Despite legislation for restriction (NETSHILUVHI, 1996; MANDER, 1998), the extraction pressure on the natural populations of these medicinal plants from the wild is still very high indicating that enforcement is incapable of curbing over-exploitation (CUNNINGHAM, 1988). With the current rate of harvesting, the plant supplies will, in time dwindle and many of the species will eventually become extinct. This will affect the rural dwellers that mostly depend on such plants (AFOLAYAN and ADEBOLA, 2004).

1.4. Conservation of medicinal plants in South Africa

In the past, professional herb gatherers and traditional healers used to collect and store plants according to their traditions. Today, the urbanised healers purchase their ingredients from the street markets and herb shops which provides an income to many otherwise unemployed people. However, this has negative effects on the environment, as this provides an incentive for the destructive harvesting of vulnerable medicinal plants (VAN WYK *et al.,* 1997).

1.4.1. Unsustainable use of medicinal plants

Demand for traditional medicines and other plant products is continuing to grow in South Africa. The ever-increasing demand for medicinal plant products results in a greater harvesting intensity on the remaining plant resources (MANDER *et al.,* 1996).

Unsustainable harvesting reduces the biodiversity of natural vegetation. This situation is illustrated by the localised extinction of popular medicinal and craftwork plants in KwaZulu-Natal (MANDER *et al*., 1996). The demand for medicinal plants exceeds supply and as a result prices have increased. According to MANDER *et al*. (1996), small *Siphonochilus* tubers can fetch over USD 2.60 and large *Bowiea* bulbs can cost over USD 5 per bulb. These prices lead to an increase in exploitation and more plant populations in other areas will be placed under harvesting pressure (MANDER *et al*., 1996).

1.4.2. Sustainable utilisation of medicinal plants

Both rural and urban populations utilise indigenous plants extensively. Plant products provide society with a wide range of essential consumer goods, including fruits, housing material, fuel wood, craftwork and traditional medicines. In addition to consumer goods, the plants provide important trade goods, with numerous enterprises being dependent on trading plant products (MANDER *et al*., 1996). The need for a sustainable supply is illustrated by the use of medicinal plants, which are frequently used by low-income groups in urban and in rural areas for primary health care (CANTER *et al.,* 2005). Therefore, there is a need to educate such communities to practice sustainable utilization of medicinal plants.

Sufficient protection of some species can be achieved through increased regulation and the introduction of sustainable wild-harvesting methods. A more viable long-term alternative is to increase domestic cultivation of medicinal plants (CANTER *et al.,* 2005). Cultivation also opens up the possibility of using biotechnology to solve problems that are inherent in the production of medicinal plants. Cultivation offers the opportunity to optimize yield and to achieve uniform, high quality production of medicinal plants (CANTER *et al.,* 2005).

VAN STADEN (1999) suggested that if the concept of sustainable use is to be effective, the population needs to be in equilibrium with the landmass it occupies. However, with South Africa‘s growing population, this equilibrium is seriously out of phase. Most of the natural populations have already been overexploited, and

those that remain are largely within conservation areas. It is these conservation areas that are targeted for sustainable harvesting. The demand for medicinal plants is already too large and meeting the demand by sustainable use would be extremely difficult (VAN STADEN, 1999).

1.5. Conservation through cultivation

Cultivation of indigenous plants could be a means of maintaining and increasing the supply of useful plants to the market (MANDER *et al.*, 1996). Recent experience has shown that it is possible to cultivate numerous indigenous plant species in sophisticated agricultural systems. However, the feasibility of cultivating these plants in small-scale, low-input farming systems is unknown (PRINSLOO *et al.,* 2007). It is important to conserve these medicinal plants by propagation and cultivation in a controlled environment.

1.6. Why cultivate medicinal plants?

The intensive harvesting of wild medicinal plants has resulted in overexploitation, and forms a serious threat to biodiversity. As a result, several plant species have been exploited to such an extent that they are seldom found in unprotected areas (CUNNINGHAM, 1991a; 1991b; WILLIAMS, 2004).

The only solution for conservation is to develop protocols for cultivating valuable medicinal plants as small-scale farming crops (VAN STADEN, 1999). This will bring sustainable development and protection of biodiversity and also generate an income for many through entrepreneurial farmers (SPARG, 2003).

Although micropropagation protocols have been established for many indigenous medicinal species, these techniques are labour-intensive, costly and therefore only feasible for high-value species (ZIV *et al.,* 1995). Seed propagation is more promising and cost-effective for the mass production of seedlings (ZHOU *et al.,* 2003).

1.7. The genus *Alepidea*

The genus *Alepidea* F. Delaroche (Apiaceae) is restricted to the African continent, with approximately twenty-eight species in southern Africa and two species in north-east Africa (BURTT, 1991; DE CASTRO and VAN WYK, 1994). According to DRUDE'S (1898) classification system for Apiaceae, *Alepidea* is placed in the subfamily Saniculoideae, together with the predominantly north-temperate genera *Eryngium* L. and *Sanicula* L., both of which contain plants of well-known medicinal value (WATT and BREYER-BRANDWIJK, 1962; STUART, 1979).

Alepidea is a genus of plants that grow in grassland (*Themeda triandra* and *Tristachya leucothrix)* areas and occurs from the Eastern Cape northwards to Mpumalanga, the Northern Province and eastern Zimbabwe (HUTCHINGS *et al*., 1996; NONJINGE and TARR, 2003). In Lesotho, it is found around Maseru (JANICE, 2002). The distribution map of *Alepidea* is shown in Figure 1.1.

1.8. *Alepidea* species

1.8.1. Distribution and morphology

Alepidea amatymbica is a perennial herb with one or more rosettes of leaves (WEIMARCK, 1949; VAN WYK *et al*., 2008). The species is characterised by basal leaves that are distinctly petiolate (VAN WYK *et al*., 2008).

It occurs at high altitudes (above 800 m) in moist areas such as stream banks and drainage zones on steep slopes, as well as on forest margins (DE CASTRO and VAN WYK, 1994). *A. amatymbica* is the most robust of all *Alepidea* species. The inflorescence is hollow, up to 1.0-1.8 m high, the rootstock is rhizomatous, up to 130 mm long and 25 mm in diameter (BURTT, 1991).

Alepidea amatymbica is distinguished from the other two smooth-fruited species; *A. macowanii* and *A. multisecta,* by its regularly dentate leaf margins lacking any deeper incision (DE CASTRO and VAN WYK, 1994). The shape of the basal

leaves and the serrations and hairs along the leaf margins are important in distinguishing the different species of *Alepidea* (VAN WYK *et al.,* 1997).

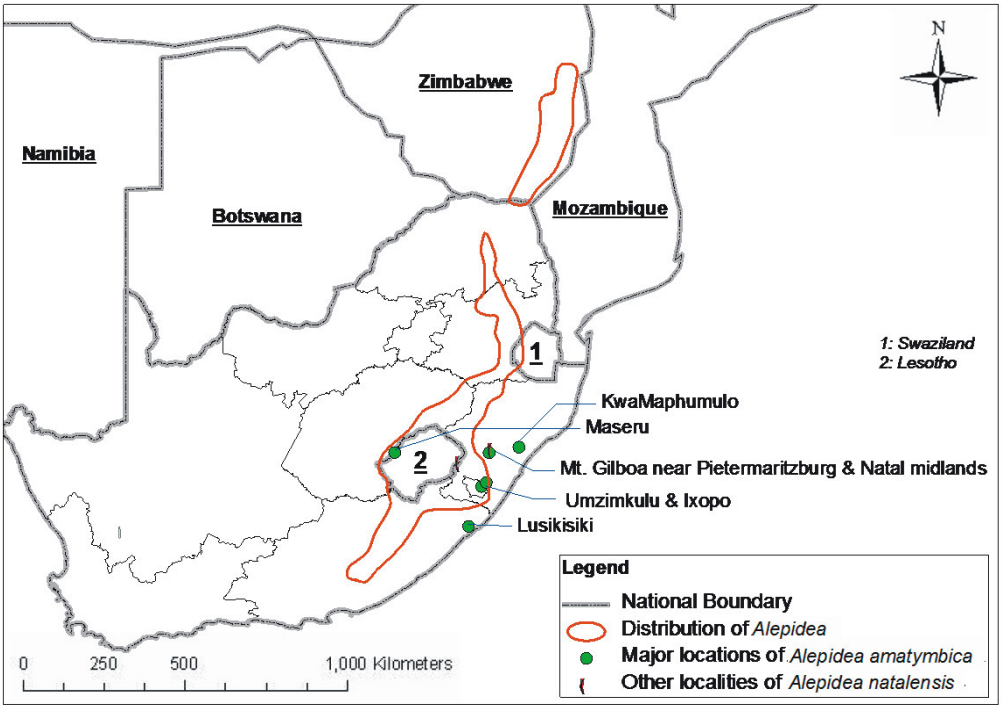

Figure 1.1: Southern African map showing distribution of the two *Alepidea* species studied in this investigation. The genus is found from the Eastern Cape, through Lesotho, KwaZulu-Natal, Mpumalanga to Limpopo, stretching to the eastern part of Zimbabwe.

The rhizome is russet-brown externally with adhering roots. The cut surface shows two rings of oleoresin ducts in a matrix of pale yellow ground tissue. The rhizome has a pleasant aromatic odour when fresh (DE CASTRO and VAN WYK, 1994).

Alepidea natalensis is distinguished from other species by its height which usually is between 200-500 mm with radical leaves in a flat rosette (DE CASTRO and VAN WYK, 1994). *A. natalensis* is found among rocks on grassland slopes. In KwaZulu-Natal, the species is found around the Drakensberg (mostly at altitudes of 1200-2300 m) and Mount Gilboa which is located to the north west of Pietermaritzburg. Its known range extends from the Cala region of the Transkei in the South, to the Harrismith area of the Orange Free State in the North

(DE CASTRO and VAN WYK, 1994). Figure 1.1 represents the geographical distribution of *Alepidea* species in South Africa.

1.8.2. Market value

There are seven species of *Alepidea* utilised for medicinal purposes: *A. amatymbica* Eckl. & Zeyh., *A. natalensis* Wood & Evans, *A. pilifera* Weimack, *A. longifolia* [E. Mey. Ex] Dümmer, *A. setifera* N.E.Br., *A. comosa* Dümmer, and *A. macowanii* Dümme. *A. amatymbica (ikhathazo* in Zulu) is the most widely utilized species. It is used by the Zulu, the Swazi, the southern Sotho and the Xhosa for medicinal purposes (WATT and BREYER-BRANDWIJK, 1962; HUTCHINGS, 1989a). *A. amatymbica* is important and is the only one of all the *Alepidea* species that is widely sold in traditional markets as *ikhathazo* in KwaZulu-Natal and *lesoko* in Lesotho (VAN WYK *et al*., 2008), and is one of the nine most popular species sold in the Durban medicinal markets (MANDER, 1998). MARSHAL (1998) reported that *A. amatymbica* is one of the most heavily traded medicinal plants in South Africa.

The price of *A. amatymbica* varies from province to province in South Africa. In KwaZulu-Natal the average market value of the dried rhizomes and roots is approximately USD 7 per kilogram (95c per rhizome) (DE CASTRO and VAN WYK, 1994). In the Eastern Cape Province mean price per kilogram is USD 12 (DOLD and COCKS, 2002).

1.8.3. Conservation status

Harvesting of rhizomes and roots usually results in plant destruction. Because of the high demand and lack of knowledge amongst communities, *A. amatymbica* is threatened due to lack of conservation knowledge (O'CONNOR, 2004).

CUNNINGHAM (1988) reported the taxon to be declining in population size in KwaZulu-Natal. MANDER (1997) ranked *A. amatymbica* var. *amatymbica* and *Siphonochilus aethiopicus* as the most sought-after medicinal plants, based on

trade popularity, in the Bushbuckridge area. MANDER (1998) estimated that 31.2 tonnes of rhizomes are traded in Durban every year. LÖTTER *et al*. (1998) reported that *A. amatymbica* was the second most popular traded medicinal plant in the Managa-Mbuzini area, in south eastern Mpumalanga. The plant is now being sourced from the highveld regions.

Alepidea amatymbica was widespread in Lesotho but now it is regarded as vulnerable. This is because it is not being used sustainably (JANICE, 2002).

1.8.4. Medicinal and other uses of *Alepidea* species

The powdered root of *A. amatymbica* is used as a snuff by diviners and healers to assist in divination and communication with 'ancestors'. The smoke obtained from burning the plant is inhaled for the same purpose. *A. amatymbica* is used by the Zulu as a remedy for colds. The Mpumalanga, Zulu and the Swazi people drink a decoction of the root to treat influenza. The Xhosa people use the plant to cure abdominal disorders and for stomach pain. A root decoction is a widespread African remedy for stomach complaints and rheumatism (GERSTNER, 1939; WATT and BREYER-BRANDWIJK, 1962). The fresh roots are chewed or the dry product is administered orally in the form of a decoction, to which honey is usually added for colds and coughs (HUTCHINGS, 1989a; DE CASTRO and VAN WYK, 1994).

In Zimbabwe, root stocks are used to treat diarrhoea, abdominal pain, and headache as well as to repel bees and as a protective charm (GELFAND *et al.,* 1985). Strained infusions of ground rootstock mixed with that of *Gunnera perpensa* L. are taken for relief of stomachache in Transkei (HUTCHINGS *et al.,* 1996). The infusion can also be mixed with *Cannabis sativa* for the treatment of asthma (VAN WYK *et al*., 1997). Smoking the roots result in mild sedation and vivid dreams. The rhizome is carried as a lucky charm (VAN WYK and GERICKE, 2000).

The leaves of both *A. natalensis* and *A. longifolia* are used as a vegetable. *A. natalensis* is known as '*ikhokwana*' by the Zulu people (FOX and YOUNG, 1988).

According to HUTCHINGS, (1989b), a mixture of *A. natalensis* and *A. longifolia* plants is used in treating gastro-intestinal diseases, respiratory diseases, venereal disease, febrile disease and snake-bites. Southern Sotho people chew the raw root or drink a decoction of the root of *A. pilifera* and *A. setifera* for chest complaints. The rhizomes of the *Alepidea* species contain resins (WATT and BREYER-BRANDWIJK, 1962).

1.8.5. Ethnopharmacology of *Alepidea* species

Sixteen species of *Alepidea* were extracted in a chemotaxonomic survey to check for kaurene derivatives (HOLZAPFEL *et al.,* 1995). Figure 1.2 shows some of the isolated kaurene derivatives. The study confirmed the presence of *ent*-9(11)-dehydro-16-kauren-19-oic acid, *ent*-16-kauren-19-oic acid, wedelia *seco*-kaurenolide and 3*β*-acetoxy derivative in the dried rhizome of *A. amatymbica* (HOLZAPFEL *et al.,* 1995). *A. amatymbica* has antimicrobial, antihypertensive and diurectic activities (HUTCHINGS, 1989a). According to BEJAR *et al.* (1984) kaurene type diterpenes exert antispasmodic and relaxant actions on smooth muscle.

Medicinal plants are commonly used in the traditional treatment of some renal diseases, and many plants are reported to possess significant diuretic activity (BELLAKHDAR, 1997; TANIRA *et al*., 1988; MAKSIMOVIC *et al.,* 2004). *A. amatymbica* was screened for potential cardiovascular and diuretic activity. All the extracts displayed low toxicity (SOMOVA *et al.,* 2001).

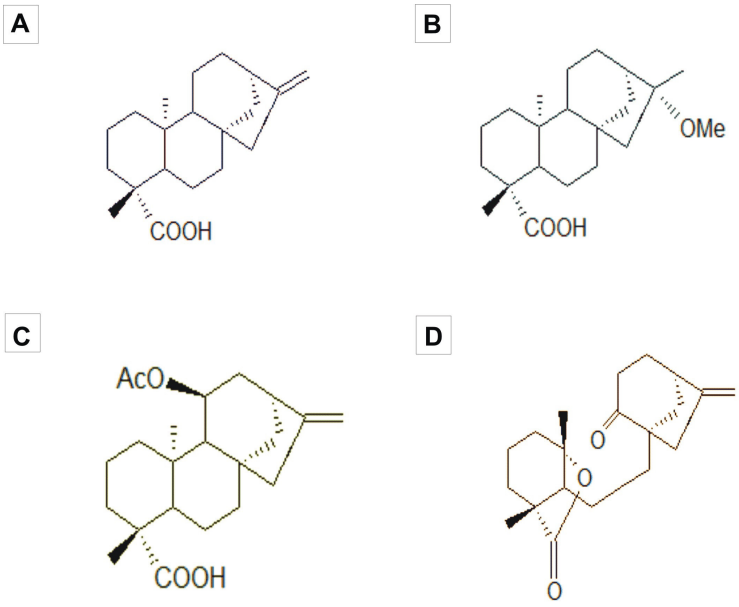

Figure 1.2: Terpenoid kaurene derivatives isolated from *A. amatymbica*. (A) *ent*-16-kauren-19-oic acid. (B) 16α-methoxy-*ent*-kaur-11-en-19-ioc-acid. (C) 11α-acetoxy-*ent*-kaur-16-en-19-oic-acid. (D) wedelia *seco*-kaurenolide adopted from . HOLZAPFEL *et al.* (1995).

1.9. Aims of the study

The large demand for *A. amatymbica* and *A. natalensis* makes them good candidates for small-scale farming. However, there is very limited documentation on the propagation practices of these two species. Although tissue culture can be a possible means to propagate these species, it remains expensive and standardization of a propagation protocol is time consuming. It was suggested that seed germination studies are key tools in conservation because they can be used for management programs and species re-introduction (ORTEGA-BAES *et al.*, 2005). Propagation through seeds therefore remains the most feasible option for conservation of these species. In spite of being valuable medicinal plants, there is very little information on seed germination of *A. amatymbica* and *A. natalensis.*

Due to the scarcity and increasing demand for *A. amatymbica*, the rhizomes of *A. natalensis* are now being uprooted as an alternative plant source. This has increased pressure on the wild populations of *A. natalensis,* and therefore there is a need for cultivation of this species also.

The study was aimed at investigating the conditions necessary for seed germination and the rational usage of the two *Alepidea* species.

To identify the environmental conditions necessary for seed germination of *A. natalensis* and *A. amatymbica*, moisture content, water uptake (imbibition percentage) as well as effects of temperature and light on seed germination were studied. After obtaining the optimal temperature and light conditions, chemical regulators, smoke-water and butenolide were evaluated to test to what extent they could improve or stimulate the germination process and seedling vigour.

The second part of the study was to investigate the medicinal properties of the two *Alepidea* species. This was done by *in vitro* testing of different extracts of the dried rhizomes of *A. amatymbica* as well as rhizomes and leaves of *A. natalensis* for antibacterial, antifungal, anti-inflammatory and genotoxicity activities.

CHAPTER 2. SEED GERMINATION STUDIES ON *Alepidea amatymbica*

2.1. Introduction

There has been a steady increase in the demand for medicinal plants since the start of the 20th century (MANDER, 1997). Many rural South Africans are dependent on medicinal plants for their primary health-care (KENDLER *et al*., 1992; CUNNINGHAM, 1994; MANDER, 1998). If the future demand for medicinal plants is to be met, it is imperative that many of the species utilised in traditional medicine be domesticated and commercially cultivated (VAN STADEN, 1999; JÄGER and VAN STADEN, 2000).

To domesticate medicinal plants, it is desirable to standardise techniques for efficient cultivation practices. In southern Africa the successful cultivation of medicinal plants is determined to a large extent by germinability of the seeds (KULKARNI *et al*., 2005a).

Alepidea amatymbica belongs to the Apiaceae family which is one of the families whose seeds have morphophysiological dormancy (MPD) (BASKIN and BASKIN, 1984). Seeds with MPD have underdeveloped, dormant embryos, which mean they have a combination of morphological and physiological dormancy. Seeds with MPD cannot be germinated unless the physiological dormancy has been broken, and embryos must grow to a certain critical length (BASKIN and BASKIN, 1998).

Seed germination is the resumption of active growth of the embryo that results in the rupture of the seed coat and the emergence of the young plant (COPELAND, 1976). The first process which occurs during germination is the uptake of water by the seed. This uptake is due to the process of imbibition. Imbibition is determined by the composition of the seed, the permeability of the seed coat or fruit to water, and the availability of water in a liquid or gaseous form in the environment (MAYER and POLJAKOFF–MAYBER, 1982).

Plants have evolved highly complex sensory mechanisms to monitor their surroundings and adapt their growth and development appropriately to the prevailing conditions (DI NOLA *et al*., 1990). Different seeds have different temperature ranges within which they germinate (MAYER and POLJAKOFF–MAYBER, 1982). The dependence of the germination rate on temperature may be one of the most important factors controlling the timing of emergence under field conditions. Germination rates determine whether a seed can germinate within a certain period of time when a combination of the environmental conditions favour germination (WASHITANI, 1985).

The optimum germination temperature for most seeds lies between 13 and 30 °C while the maximum temperature for seed survival for most species is from 35 to 40 °C (COPELAND, 1976). However, according to a study by SPARG *et al.* (2005a), germination of *Merwilla natalensis* seeds was observed under 10 °C and above 40 °C. Work done by KULKARNI *et al.* (2007) on germination of *Dioscorea dregeana* under different temperature regimes showed that there was no germination at 10 °C.

Light is one of the most important environmental factor regulating growth and development of plants. When absorbed by chlorophyll, light represent a source of energy (MANCINELLI, 1994). Since the mid-19th Century, light has been recognized as a germination-controlling factor (BASKIN and BASKIN, 1998). MAYER and POLJAKOFF-MAYBER (1989) suggested that light-sensitivity of seed has an influence on seed germination in their natural habitat.

Seeds may be divided into two groups, those which germinate under dark conditions and those that need light (MEYER and POLJAKOFF–MAYBER, 1982). Seed of *Tulbaghia alliacea* and *Dianthus basuticus* have been reported to germinate well in both light and dark conditions. *Urginea capitata* seeds also germinated well with the continuous light treatment (KULKARNI *et al.,* 2005a).

Relatively few species have seeds that germinate better in darkness than in light (BASKIN *et al*., 1989). The seeds of *Albuca pachychlamys* were reported to germinate at a higher percentage (100%) under constant dark conditions,

compared to 87.5% germination under constant light. In the germination study of *Drimia robusta*, the opposite effect was observed with 93% germination observed under constant light and 80% in the dark (KULKARNI *et al.,* 2005b).

2.1.1. Objectives of this study

The objectives of this study were to determine the optimal temperature and light conditions for seed germination of *A. amatymbica* as well as to improve germination by the use of smoke solution and chemical substances.

2.2. Materials and Methods

2.2.1. Seed collection

Seeds of *A. amatymbica* used in this study were collected between February and March 2004 from Maseru (29°18´S, 27°30´E), Lesotho. After cleaning, the seeds were stored in a sealed glass bottle at -20 °C for 3 years before being used in the seed germination experiments.

2.2.2. Seed viability

The seed viability of the collected *A. amatymbica* seeds was tested using a 2,3,5-triphenyl tetrazolium chloride (TTC) solution (ISTA, 1999). Seeds were soaked in a 1% solution of TTC for 24 h at 25 ± 2 °C in the dark. Seeds were then cut longitudinally and the red-stained embryos were considered as representing viable seeds.

2.2.3. Seed moisture content and imbibition

Moisture content of the seeds was determined by drying them at 110 °C in a preset incubator until there was no further loss in seed weight. Moisture content, expressed as percentage, was calculated on the basis of fresh weight using the equation below (BEWLEY and BLACK, 1982):

$$\text{Moisture content (\%)} = \left(\frac{\text{Fresh weight of seed} - \text{Dry weight of seed}}{\text{Fresh weight of seed}}\right) \text{X } 100$$

In imbibition studies, four replications of 20 seeds were placed in 6.5 cm disposable Petri dishes on two layers of filter paper (Whatman No.1) moistened with 3 ml distilled water and allowed to imbibe at room temperature (25 ± 0.5 ℃). The increase in seed mass was determined after 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h. At each interval, the seeds were blotted dry, weighed and then replaced on the wet filter papers. The percentage increase was plotted graphically.

2.2.4. Germination studies

The seeds of *A. amatymbica* were placed on two layers of Whatman No.1 filter paper in disposable plastic Petri dishes (6.5 cm). The filter paper was moistened with 3 ml distilled water or test solutions and kept wet by adding water or the respective test solutions when required. The seeds were not decontaminated. For each experiment, there were four replicates of 25 seeds each. The standard experimental conditions were a 16 h photoperiod provided by cool-white fluorescent lamps with photosynthetic photon flux density (PPF) of 90.5 µmol m^{-2} s^{-1} at 25 ℃.

Experiments were terminated after 4 weeks. MGT was calculated by using the equation:

$$\textbf{MGT} = \sum \frac{(n \times d)}{N}$$

where n = number of seeds germinated on each day, d = number of days from the beginning of the test and N = total number of seeds germinated at the termination of the experiment (ELLIS and ROBERTS, 1981).

2.2.4.1. Effect of temperature

To evaluate the effects of different temperature regimes, the seeds were incubated at constant temperatures (10, 15, 20, 25, 30, and 35 °C) and an alternating temperature of 30 /15 °C under a 16 h photoperiod regime.

2.2.4.2. Effect of photoperiod

To evaluate the effects of photoperiod, seeds were germinated under constant light supplied by cool-white fluorescent lamps (PPF of 40.5 µmol $m^{-2}s^{-1}$), alternating light (16 h photoperiod) and constant dark where Petri dishes were kept in light-proof boxes and germination was recorded under a green safe light (0.3 µmol $m^{-2}s^{-1}$) at 25 ± 2 °C daily.

2.2.4.3. Effect of cold stratification

Stratification treatments consisted of seeds being placed between two sheets of moist paper towels in a plastic bags at 5 °C for 7, 14, 21, 28 and 35 days. Subsequently, the seeds were germinated under light conditions (16 h photoperiod) at 25 °C. Non-stratified seeds were considered as the control.

2.2.4.4. Effect of sowing depth

Three replicates of 10 seeds each were sown in plastic pots (10 cm) at depths of 0.5, 1.0, 1.5, 2.0 and 2.5 cm in sterile quartz sand moistened with 100 ml distilled water. The experimental pots were randomly placed in a greenhouse. Seedling emergence above the soil surface was recorded daily for a period of 28 days.

2.2.4.6. Effect of smoke solutions

Seeds were incubated under alternating light (16 h photoperiod) with smoke-water (dilution of 1:500 v/v) or smoke-derived butenolide (3-methyl-*2H*-furo[2,3-c]pyran-2-one, 10^{-7}, 10^{-8} and 10^{-9} M) isolated from smoke (VAN STADEN *et al*., 2004). The smoke-water was prepared by the methods outlined by BAXTER *et al*. (1994).

2.2.4.7. Effect of chemical substances

Chemical substances tested on seed germination were kinetin, gibberellic acid (GA_3) and potassium nitrate (KNO_3) at concentrations of 10^{-6}, 10^{-7} and 10^{-8} M under alternating light/dark (16 h photoperiod) conditions at 25 ℃.

2.2.5. Statistical analysis

Percentage germination data were arcsine transformed before analysis (ZAR, 1996). One-way analysis of variance (ANOVA) was conducted using MINITAB® release 14 statistical package (Minitab Inc., Chicago, USA), and Tukey's test was evaluated at the 5% level of significance.

2.3. Results and Discussion

2.3.1. Moisture content, seed viability and water uptake

The moisture content of *A. amatymbica* seeds was found to be 4% after three years of storage. A quick and easy method of checking the viability of seeds is the tetrazolium test (COTTRELL, 1947; PETERS, 2000). The seed viability of *A. amatymbica* species was 92% after 3 years of storage. The seeds of *A. amatymbica* imbibed water to a maximum after 72 h (Fig. 2.1).

Seeds will not germinate unless both minimal soil water potential and seed hydration are attained during imbibition (HADAS and RUSSO, 1974). Water is therefore an essential component for the rehydration and germination of seeds (BEWLEY and BLACK, 1978). In this study, seeds of *A. amatymbica* that had been stored for 3 years showed high imbibition of water. This can be attributed to the low moisture content and the age of the *A. amatymbica* seeds.

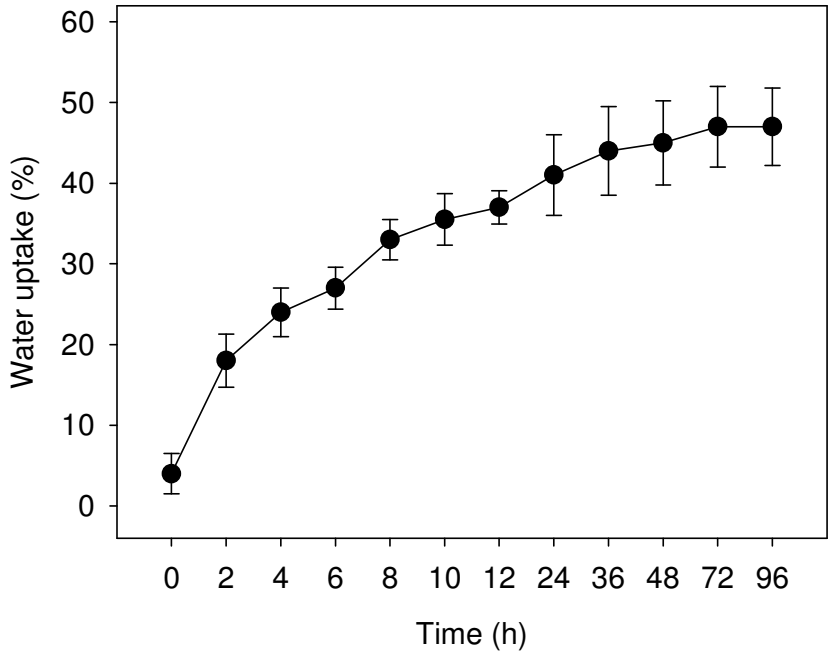

Figure 2.1: Water uptake by seeds of *A. amatymbica* at 25 ℃ under a 16 h photoperiod.

2.3.2. Temperature and light requirements for seed germination

2.3.2.1. Effect of temperature

At a temperature of 25 ℃, seeds of *A. amatymbica* exhibited maximum percentage germination (72.5%) with a shorter MGT when compared to 10 and 15 ℃ and alternating temperatures 30/15 ℃ (Fig. 2.2). In comparison to the other temperatures, significantly lower percentage germination was recorded at 30 and 35 ℃ (8.7 and 0%, respectively). High temperatures may prevent germination because proteins may be denatured and the permeability of the membranes altered, while at low temperatures there is reduced metabolic activity which prevents the germination processes (DELACHIAVE and DE PINHO, 2003).

The optimum temperature of *A. amatymbica* seed germination is 25 ℃. This result shows that seeds of *A. amatymbica* are temperature dependent for germination.

Temperature is one of the most important environmental factors in controlling seed germination (ARMITAGE, 1994). According to HARTMANN *et al*. (1997a), alternating temperature often results in better germination than constant temperatures. However, in this study alternating temperature (30/15 ℃) did not significantly improve germination (Fig. 2.2).

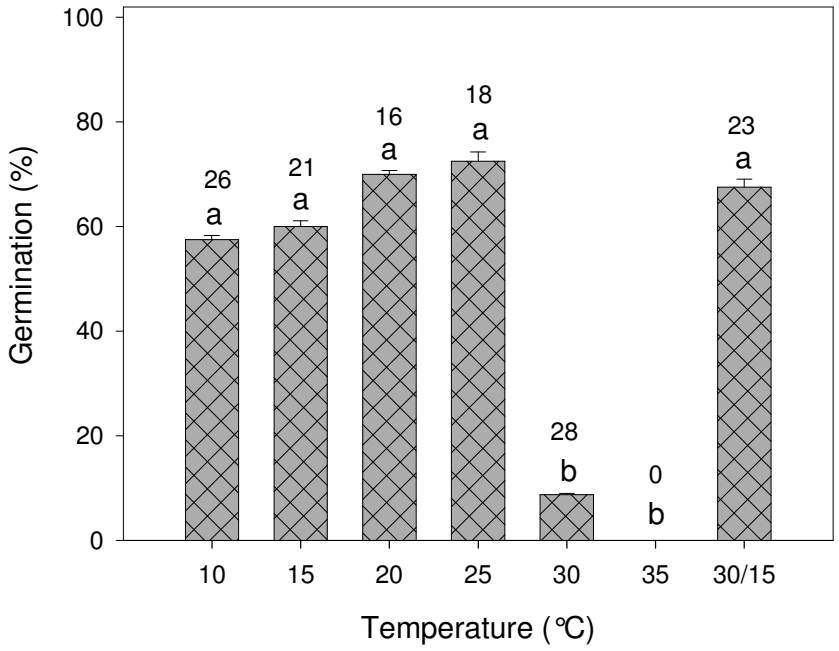

Figure 2.2: Effect of different temperatures on seed germination of *A. amatymbica* under a 16 h photoperiod. Bars (± SE) with different letters are significantly different ($p < 0.05$). The number above the letters represents MGT in days.

2.3.2.2. Effect of photoperiod

Under alternating and constant light conditions, the seeds of *A. amatymbica* showed significantly higher percentage germination (72.5% and 60%, respectively) with a shorter MGT when compared to constant dark conditions (36.2%) (Fig. 2.3). However, the percentage germination under constant light was not significantly different to alternating light conditions.

Light-sensitivity of seeds is suggested to have some relation to seed germination in their natural habitat (MAYER and POLJAKOFF-MAYBER, 1989). In this study, germination of *A. amatymbica* seeds was significantly higher under constant light compared to constant dark conditions (Fig. 2.3). Similar findings were reported by KULKARNI *et al.* (2005a) for germination of *Urginea capitata* where continuous light treatment resulted in the highest germination (100%).

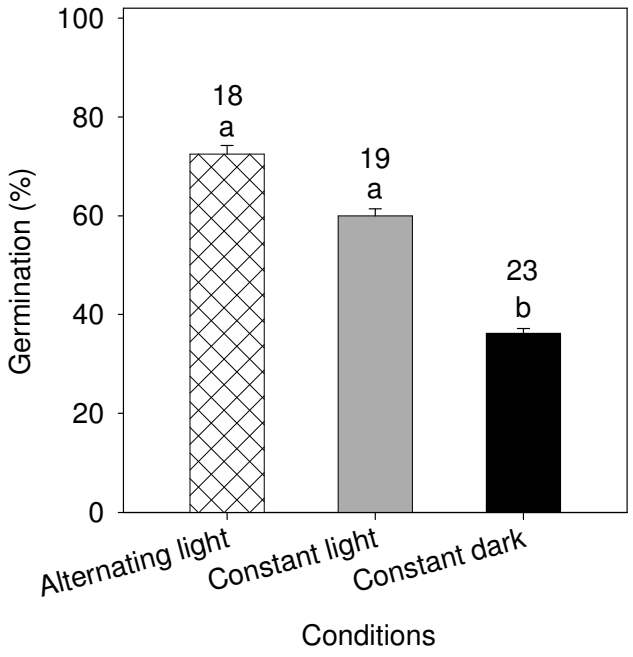

Figure 2.3: Effect of different light conditions on seed germination of *A. amatymbica* at 25 ℃. Bars (± SE) with different letters are significantly different ($p < 0.05$). The number above the letters represents MGT in days.

2.3.3. Effect of sowing depth

The effect of sowing depth on seed germination of *A. amatymbica* is shown in Table 2.1. At a depth of 0.5 cm 40% of the seeds germinated whereas 33% germination was obtained at a depth of 1 cm. Poor germination was recorded at a depth of 2.5 cm. Seeds of *Nardostachys jatamansi* Sowed at a depth of 0.5 cm showed the highest germination percentage (CHAUHAN and NAUTIYAL, 2007). Sowing of *Merwilla natalensis* seeds at depths of 1.0 and 2.0 cm resulted in higher percentage germination than at depths of 3.0 and 4.0 cm (SPARG *et al*., 2005a). From this result, it seems that the seeds of *A. amatymbica* must be sown near the soil surface. In the wild, this species occur on the slopes of mountain and rocky places (O'CONNOR, 2004), where there is little chance of deep burial.

Table 2.1: Effect of sowing depth on seed germination of *A. amatymbica*.

Sowing depth (cm)	Germination (%)	MGT (days)
0.5	40 ± 1.0 a	13 ± 1.0 a
1.0	33 ± 1.0 a	14 ± 1.0 a
1.5	25 ± 0.0 a	9 ± 3.0 ab
2.0	5.0 ± 1.0 b	10 ± 4.0 a
2.5	3.0 ± 0.0 b	2 ± 2.0 c

MGT= mean germination time. Mean values (± SE) in a column with different letters are significantly different ($p < 0.05$).

2.3.4. Effect of cold stratification

Fourteen days of stratification of *A. amatymbica* seeds significantly improved germination (91.2%) when compared with non-stratified (control) seeds (72.5%) (Fig 2.4). It is suggested that seeds that are exposed to adverse winters require longer periods of cold stratification for germination than those exposed to milder winters (CAVIERES and ARROYO, 2000). This also depends on the altitude at which the plants occur. Seeds from high elevation populations could require longer periods of cold stratification to germinate (CAVIERES and ARROYO, 2000). It is reported that stratification eliminates the blocking of GA_3 biosynthesis, which is activated when the seed is transferred to higher temperatures (BRADBEER *et al*., 1978). This study shows that stratification is needed to enhance germination of

this species. Similar results were reported by BAKER *et al*. (2005) where cold stratification induced seed germination of *Actinotus leucocephalus,* another member of the Apiaceae.

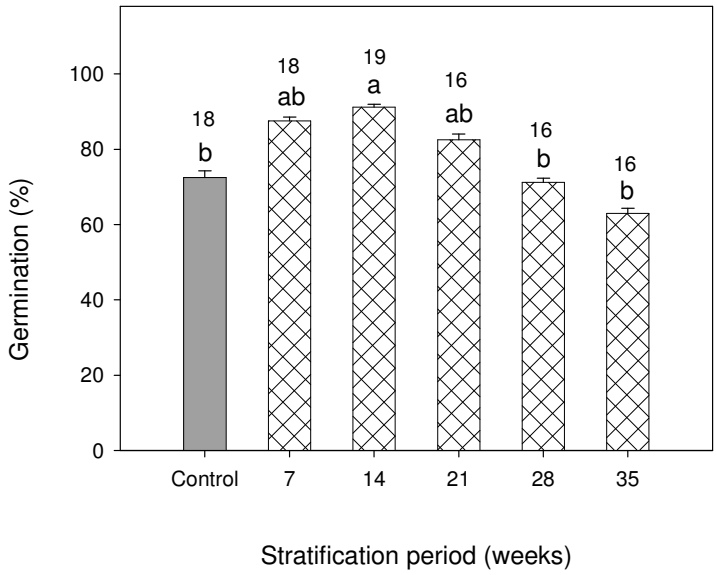

Figure 2.4: Effect of different cold stratification (5 ℃) periods on seed germination of *A. amatymbica* at 25 ℃ under a 16 h photoperiod. Bars (± SE) with different letter(s) are significantly different ($p < 0.05$). The number above the letters represents MGT in days.

2.3.4. Effect of smoke solution and chemical substances

Seeds of *A. amatymbica* incubated with smoke-water (1:500 v/v) under alternating light (16 h photoperiod) at 25 ℃ showed a significantly higher percentage germination (91.2%) than the control seeds (65%). Treatments with butenolide isolated from smoke, GA_3, KNO_3 and kinetin did not significantly increase germination of *A. amatymbica* but shortened MGT in comparison to the control (Table 2.2).

Table 2.2: Effect of smoke solutions and chemical substances on seed germination of *A. amatymbica* at 25 ℃ under a 16 h photoperiod.

Treatment	Germination (%)	MGT (days)
Control	65.0 ± 1.4 b	22.0 ± 1.5 a
GA_3 (10^{-6}M)	60.0 ± 1.6 b	18.7 ± 1.2 bc
GA_3 (10^{-7}M)	71.2 ± 0.7 b	19.0 ± 0.4abc
GA_3 (10^{-8}M)	72.5 ± 1.1 b	20.2 ± 0.4 ab
Kinetin (10^{-6}M)	61.2 ± 1.3 b	19.5 ± 1.0 abc
Kinetin (10^{-7}M)	63.7 ± 0.8 b	21.0 ± 0.8 ab
Kinetin (10^{-8}M)	63.0 ± 1.4 b	18.7 ± 1.0 bc
KNO_3(10^{-6}M)	68.0 ± 1.0 b	15.0 ± 1.0 c
KNO_3(10^{-7}M)	53.0 ± 1.0 b	15.0 ± 1.0 c
KNO_3(10^{-8}M)	55.0 ± 1.0 b	16.0 ± 1.0 c
Butenolide (10^{-7}M)	65.0 ± 1.4 b	15.0 ± 1.0 c
Butenolide (10^{-8}M)	65.0 ± 1.0 b	17.0 ± 0.4 c
Butenolide (10^{-9}M)	66.0 ± 2.0 b	17.0 ± 0.2 c
Smoke-water (1:500 v/v)	91.2 ± 0.7 a	17.2 ± 0.2 c

MGT = mean germination time. Mean values (± SE) in a column with different letters are significantly different ($p < 0.05$).

Smoke-treatments have shown positive results in promoting seed germination and seedling vigour in a number of medicinal plants (SPARG *et al*., 2005b; KULKARNI *et al*., 2006). In this study, smoke-water treatment was effective in promoting germination of *A. amatymbica* seeds. This shows that smoke-water treatments can be used to increase percentage germination of *A. amatymbica.*

Smoke-water stimulated the percentage germination of long-term stored seeds. This result confirms that seeds of this species can remain viable after 3 years of storage. Germination studies on 1- and 7-year-old stored seeds of *Hakea dohertyi* suggested that long-term storage of seed has an advantage for conservation of plants (OFFORD *et al.,* 2003).

2.4. Conclusions

The seeds of *A. amatymbica* require alternating light conditions for optimum germination. The most favourable temperature for germination of the species is 25 °C. Cold stratification (5 °C) for 2 weeks improved percentage germination of *A. amatymbica*. Smoke-water (1:500 v/v) was beneficial in enhancing percentage germination of *A. amatymbica* seeds. The best depth for sowing seed of this species is near the soil surface. It is suggested that different stratification treatments can be used to break dormancy. This study showed that temperature, light conditions, cold stratification, a smoke solution and chemical substances can break physiological dormancy after storing *A. amatymbica* for three years. The results can be useful in optimizing the germination of *A. amatymbica* for large-scale propagation practices.

CHAPTER 3. SEED GERMINATION STUDIES ON *Alepidea natalensis*

3.1. Introduction

The seed is the structure in which a fully developed plant embryo is dispersed. It enables the embryo to survive until seed maturation and seedling establishment, thereby ensuring the initiation of the next generation (KOORNNEEF *et al*., 2002). BEWLEY and BLACK (1994) defined germination as the events that commence with the uptake of water by the quiescent dry seed and terminates with the elongation of the embryonic axis.

Different seeds have a range of temperatures at which germination will occur optimally, below and above which germination is delayed but not prevented (MAYER and POLJAKOFF-MAYBER, 1982). The optimal temperature may be taken to be that at which the highest percentage of germination is attained in the shortest time (MAYER and POLJAKOFF-MAYBER, 1982).

Light is one of the most important environmental factors that interact with temperature to regulate seed germination in many plant species (BASKIN and BASKIN, 1998), but light requirement for germination may vary with temperature (EL-KEBLAWY and AL-RAWAI, 2005). There are other factors such as cold stratification that can influence the germination percentage. KULKARNI *et al.* (2006) observed an increase in the germination percentage of *Eucomis autummalis* after storage of seed under cold stratification (5 ℃) for 45 days. Several studies have reported on seed germination with different temperatures, light conditions and different treatments for seeds of medicinal plants of southern Africa (KULKARNI *et al.,* 2005 a, b; 2006; 2007; SPARG *et al*., 2005a).

Many plant species are under threat, and are often difficult to find outside protected areas. Some of these species are now imported from neighbouring countries. However, it will not be long before the wild populations in these

countries get depleted (VAN STADEN, 1999; JÄGER and VAN STADEN, 2000). It has been suggested that domestication and commercial cultivation could be very important to species utilized in traditional medicine (VAN STADEN, 1999; JÄGER and VAN STADEN, 2000).

Smoke treatments show promise in the propagation of economically important wild plants, as in many plant species germination percentage can be increased significantly (BROWN and VAN STADEN, 1998; BROWN and BOTHA, 2004). Smoke and smoke solutions have great potential for use in horticulture, agriculture, weed management, habitat restoration and conservation practices (ROCHE *et al*., 1997; BOUCHER and MEETS, 2004; LIGHT and VAN STADEN, 2004).

The compound that has been isolated from smoke plays an active role in stimulating seed germination of many plants. The compound has been characterized as the butenolide (3-methyl-*2H*-furo[2,3-c]pyran-2-one), isolated from plant-derived smoke (VAN STADEN *et al*., 2004), burned cellulose (FLEMATTI *et al*., 2004), and also from products formed by heating combinations of carbohydrates and amino acids (LIGHT *et al*., 2005). This compound is now recognized as a germination cue for many smoke-exposed and smoke non-exposed naturally occurring species, showing a good potential as a stimulant for germination of agricultural and horticultural crops (KULKARNI *et al*., 2008).

Smoke has been shown to release seed dormancy and to improve germination. A few studies have shown that the effects of smoke extend beyond post-germination events resulting in the stimulation of seedling vigour (BAXTER and VAN STADEN, 1994; SPARG *et al*., 2005a).

In studies with *Dioscorea dregeana,* butenolide and smoke-water were effective in promoting germination and shortening the MGT (KULKARNI *et al.,* 2007). Smoke–water and butenolide had a promotive influence on *Eucomis autumnalis* seed germination (KULKARNI *et al*., 2006). In a study by SPARG *et al*. (2005b), smoke improved seedling vigour of some South African indigenous medicinal plants.

Seed dormancy can be broken by many chemical substances, which include chemical regulators such as GA_3, GA_4, GA_7, kinetin, BA, nitrogen compounds (KNO_3 and $NaNO_2$), potassium permanganate ($KMnO_4$) and peroxides (H_2O_2). Effects of these chemicals are often a function of the concentration and treatment durations. However, seed germination of some plant species does not respond to these regulators (BEWLEY and BLACK, 1994; FONTAINE *et al*., 1994; WANG *et al*., 1996).

Plant regulators such as GA_3 and IAA (HILHORST and KARSSEN, 1992; IGLESIAS and BABIANO, 1997) and chemicals such as KNO_3 (KEVSEROĞLU, 1993; HARTMANN *et al*., 1997b) have been recommended to break dormancy and enhance germination (CIRAK *et al*., 2007). The stimulatory effect of GA_3 on germination of dormant seed has been reported for many plant species, such as lettuce (*Lactuca sativa*) (MAYER and POLJAKOFF-MAYBER, 1982).

Nitrogen compounds can break seed dormancy by decreasing carbon monoxide (CO) and changing metabolic pathways, thus they are usually used as a germination accelerator (BEWLEY and BLACK, 1994). A study by YANG *et al.* (2007) using KNO_3 and $NaNO_3$ showed accelerated germination of *Areca triandra* in such a way that germination was faster at higher concentrations. Potassium nitrate is one of the frequently used germination stimulants. PALANI *et al.* (1995) reported an increase in germination from 37% to 79% in a study of *Acacia nilotica* after treatments with KNO_3.

3.1.1. Objectives of this study

The objective of the study were to determine the optimal temperature and light condition for *A. natalensis* seed germination, the influence of smoke solutions and chemical substance on seed germination of *A. natalensis* seed, and to improve seedling vigour using smoke solutions.

3.2. Materials and Methods

3.2.1. Seed collection

Fresh *A. natalensis* (Wood & Evans) seeds were collected between March and February 2007 from Mount Gilboa (29°16.766´S, 30°17.627´E) near Pietermaritzburg in the KwaZulu-Natal Province, South Africa. These seeds were cleaned and stored in brown seed packets at room temperature (25 ± 2 ℃) for a period of 30 days before conducting seed germination experiments.

3.2.2. Seed viability

The seed viability of the collected *A. natalensis* seeds was tested using a 2,3,5-triphenyl tetrazolium chloride (TTC) solution (ISTA, 1999). Seeds were soaked in a 1% solution of TTC for 24 h at 25 ± 2 ℃ in the dark. Subsequently, seeds were longitudinally cut and the red-stained embryos were considered as representing viable seed.

3.2.3. Seed moisture content and imbibition

Moisture content of the seeds was determined by drying them at 110 ℃ in a preset incubator until there was no further loss in seed weight. Moisture content, expressed as percentages, was calculated on the basis of fresh weight using the equation below (BEWLEY and BLACK, 1982):

$$\text{Moisture content } (\%) = \left(\frac{\text{Fresh weight of seed} - \text{Dry weight of seed}}{\text{Fresh weight of seed}}\right) \text{X } 100$$

In imbibition studies, four replications of 20 seeds were placed in 6.5 cm disposable Petri dishes on two layers of filter paper (Whatman No.1) moistened with 3 ml distilled water and allowed to imbibe at room temperature (25 ± 0.5 ℃). The increase in seed mass was determined after 2, 4, 6, 8, 12, 24, 36, 48, 72 and

96 h. At each interval, the seeds were blotted dry, weighed and then replaced on the wet filter papers. The percentage increase was plotted graphically.

3.2.4. Germination studies

The seeds of *A. natalensis* were placed on two layers of Whatman No.1 filter paper in disposable plastic Petri dishes (6.5 cm). The filter paper was moistened thoroughly with 3 ml distilled water or test solutions and kept moist by adding water or respective test solutions when required. The seeds were not decontaminated. For each experiment, there were four replicates of 25 seeds each. The standard experimental conditions were a 16 h photoperiod provided by cool-white fluorescent lamps and PPF of 90.5 µmol $m^{-2}s^{-1}$ at 25 °C. Experiments were terminated after 4 weeks. MGT was calculated by using the equation:

$$\mathbf{MGT} = \sum \frac{(n \times d)}{N}$$

where n = number of seeds germinated on each day, d = number of days from the beginning of the test, and N = total number of seeds germinated at the termination of the experiment (ELLIS and ROBERTS, 1981).

3.2.4.1. Effect of temperature

To evaluate the effects of different temperature regimes, the seeds were incubated at constant temperatures (10, 15, 20, 25, 30, and 35 °C) and an alternating temperature of 30/15 °C under a 16 h photoperiod. The higher temperature coincided with the light period. Seeds which failed to germinate at constant temperatures over 21 days were subsequently shifted to the next higher temperature.

3.2.4.2. Effect of photoperiod

To evaluate the effect of photoperiod, seeds were germinated under constant light supplied with cool-white fluorescent lamps (PPF of 40.5 µmol $m^{-2}s^{-1}$), alternating

light (16 h photoperiod) as well as constant dark where Petri dishes were kept in light-proof boxes and germination was recorded under a green “safe light” (0.3 µmol $m^{-2}s^{-1}$) at 25 ± 2 ℃ daily.

3.2.4.3. Phytochrome effects

Seeds were imbibed in the dark at 25 ± 0.5 ℃ for 1 h before being exposed to different light filters for 10 min each. Light filters used were red light (1.5 µmol $m^{-2}s^{-1}$), far-red (1.4 µmol m $m^{-2}s^{-1}$), alternating red to far-red light, far-red to red light, red to far-red to red, far-red to red to far-red. After exposure to light filters, all the seeds were placed in light-proof boxes and incubated under continuous dark at 25 ℃. White light and dark incubation were used as control. Germination was recorded under a green “safe light”.

3.2.4.4. Effect of cold stratification

Stratification treatments consisted of seeds being placed between two sheets of moist paper towels in plastic bags at 5 ℃ for 7, 14, 21, 28 and 35 days. Subsequently, the seeds were germinated under light conditions (16 h photoperiod) at 25 ℃. Non-stratified seeds were considered as the control.

3.2.4.5. Seed storage study

Four replicates of 25 seeds each were stored for 6 month at -20 ℃, 5 ℃ and room temperature (25 ℃). After the desired period of storage, germination tests were performed as outlined above at 25 ± 0.5 ℃. The seeds were incubated for 28 days. Germination counts were conducted daily. Seeds were considered germinated when the radicle protruded about 2 mm in length.

3.2.4.6. Effect of sowing depth

Three replicates of 10 seeds each were sown in pots (10 cm) at depths of 0.5, 1, 1.5, 2.0 and 2.5 cm in sterile quartz sand and moistened with 100 ml distilled water. The experimental pots were randomly placed in a green house. Seedling emergence above the soil surface was recorded daily for a period of 28 days.

3.2.4.7. Effect of smoke solutions

Seeds were incubated with smoke-water (dilution of 1:500 v/v) or smoke-derived butenolide (3-methyl-*2H*-furo[2,3-c]pyran-2-one, 10^{-7}, 10^{-8} and 10^{-9} M) isolated from smoke (VAN STADEN *et al*. 2004). The smoke-water was prepared by the methods outlined by BAXTER *et al*. (1994).

3.2.4.8. Effect of chemical substances

Chemical substances tested on seed germination were kinetin, gibberellic acid (GA_3) and KNO_3 at concentrations of 10^{-6}, 10^{-7} and 10^{-8} M under alternating light/dark (16 h photoperiod) conditions at 25 °C.

3.2.4.9. Effect of smoke solutions under different temperature and different light conditions

To determine the effect of different temperatures and light conditions, four replicates of 10 seeds each were incubated at 10, 15, 20, 25, and 30 °C. The experiments were conducted under a 16 h photoperiod, with cool-white fluorescent lamps, which provided a (PPF of 80.6 ± 7.8 µmol $m^{-2}s^{-1}$). For continuous dark treatments, the Petri dishes were placed in light-proof wooden boxes at 25 ± 0.5 °C, and germination was assessed daily under a green “safe light” (0.3 µmol $m^{-2}s^{-1}$). For continuous light, the seeds were exposed to a PPF of 40.5 µmol $m^{-2}s^{-1}$. In the case of smoke treatments, the experiments were terminated after seven weeks and seedling growth assessed. Seedling vigour indices (SVI) were then calculated using the following formula; SVI = seedling length (mm) X germination (%) (ABDUL-BAKI and ANDERSON, 1973).

3.2.5. Statistical analysis

Percentage germination data were arcsine transformed before analysis (ZAR, 1996). One-way analysis of variance (ANOVA) was conducted using MINITAB® release 14 statistical package (Minitab Inc., Chicago, USA), and Tukey's test was evaluated at 5% level of significance.

3.3. Results and Discussion

3.3.1. Moisture content, seed viability and water uptake

The moisture content of freshly harvested seed of *A. natalensis* was 7%. The seed viability of *A. natalensis* species was found to be 92%. The seeds of *A. natalensis* imbibe water to the maximum after 96 h (Fig. 3.1). Imbibition differs greatly between seeds in relation to seed size and weight, structure, permeability, chemical composition, variation in temperatures and seed-water contact areas (OBROUCHEVA and ANTIPOVA, 1997).

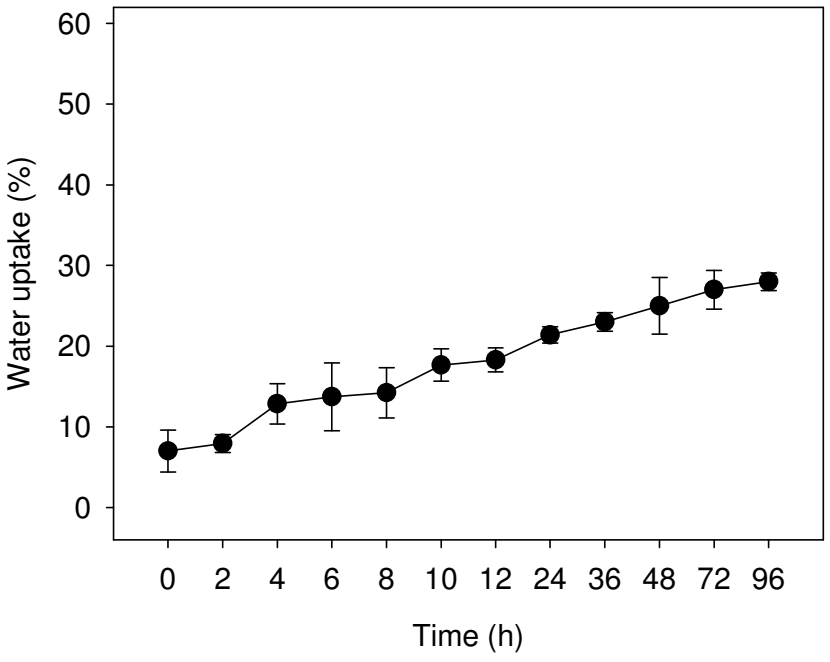

Figure 3.1: Water uptake by seeds of *A. natalensis* at 25 ℃ under a 16 h photoperiod.

3.3.2. Effect of temperature

Alepidea natalensis seeds incubated at 15-25 ℃ showed significantly higher percentage germination in comparison to other temperatures examined (Fig. 3.2). However, the MGT was longer for the seeds germinated at 15 ℃ compared to 20 and 25 ℃. At low (10 ℃) and high temperatures (30 ℃) seeds of *A. natalensis* showed significantly lower percentage germination than at 25 ℃. At 35 ℃ no seeds germinated.

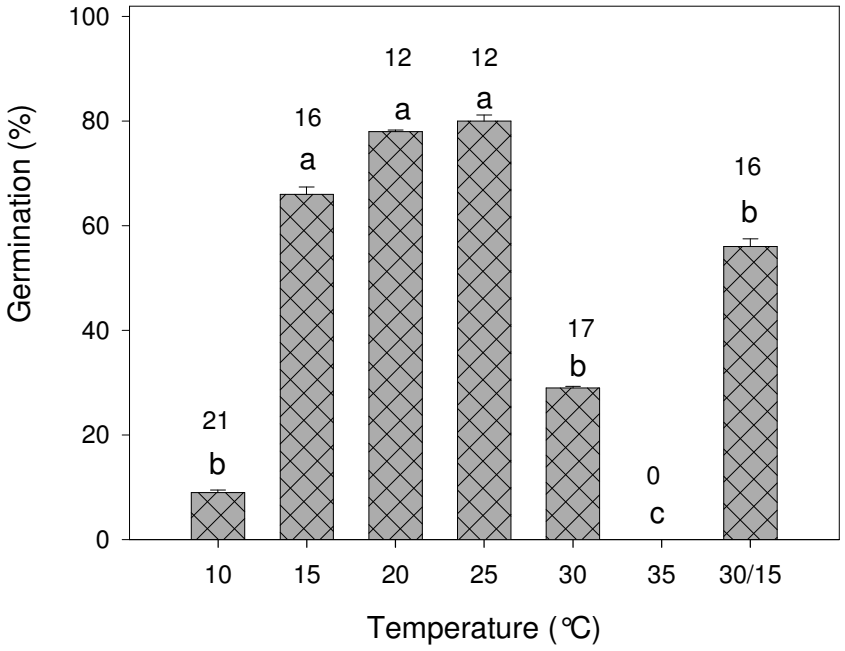

Figure 3.2: Effect of different temperatures on seed germination of *A. natalensis* under a 16 h photoperiod. Bars (± SE) with different letters are significantly different ($p < 0.05$). The number written above the letters represents MGT to reach maximum germination in days.

Seed germination was completely inhibited at 35 ℃. This shows that the seeds of *A. natalensis* cannot tolerate high temperatures. According to BASKIN and BASKIN (1998) temperatures unfavourable for germination may delay seed germination. At alternating temperatures (30/15 ℃), 66% germination was

obtained. The temperature that achieved the highest germination with the shortest MGT was 25 ℃, suggesting that 25 ℃ is the optimum temperature for seed germination of this species. Similarly, a tuberous medicinal plant *Dioscorea dregeana* showed the highest percentage germination at 25 ℃ in comparison to the other constant temperatures examined (KULKARNI *et al*., 2007).

3.3.3. Effect of photoperiod

There was a significantly higher germination rate of *A. natalensis* seeds incubated under alternating and constant light conditions (80% and 73%, respectively). Seeds incubated under constant dark conditions had a lower germination (29%) and longer MGT. No significant difference in germination was observed between alternating light conditions and constant light.

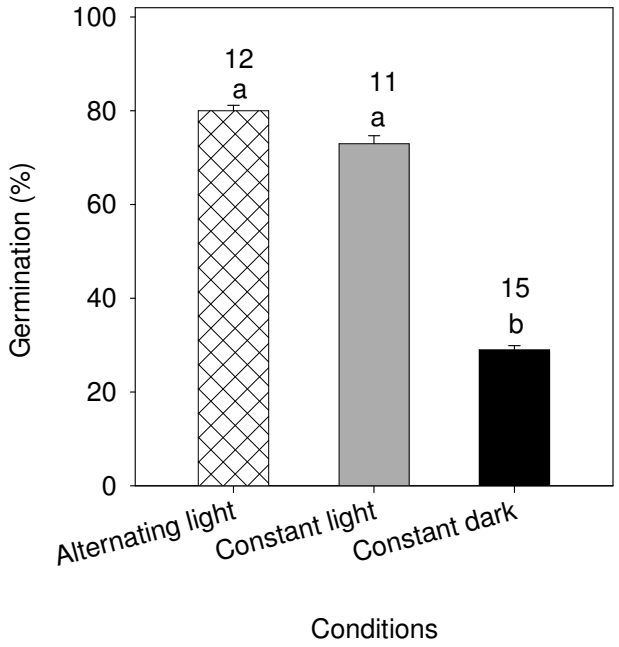

Figure 3.3: Effect of different light conditions on seed germination of *A. natalensis* at 25 ℃. Bars (± SE) with different letters are significantly different ($p < 0.05$). The number written above the letters represents MGT in days.

Light and temperature play a significant role in seed germination of many plant species (BEWLEY and BLACK, 1982). Under alternating and constant light

conditions at 25 °C, *A. natalensis* achieved maximum germination. In contrast, percentage germination of these seeds was inhibited under constant dark conditions. This indicates that *A. natalensis* is a light requiring species and can be considered as positively photoblastic. Seeds of *Drimia robusta*, a bulbous medicinal plant, showed higher percentage germination when incubated under constant light rather than dark conditions (KULKARNI *et al*., 2005a).

3.3.4. Effect of temperature shifts

Seeds germinate over a wide range of temperatures, but the maximum and minimum temperatures for germination vary with each species (BASKIN and BASKIN, 1998). At 10 °C, there was a delay in germination of *A. natalensis* seeds, whilst at 30 °C there was low percentage germination (Fig. 3.2). The seeds which had not germinated at 10 °C and 30 °C were shifted to 25 °C after 21 days. Temperature shift from 10 °C exhibited a stimulatory effect on seed germination of *A. natalensis* seeds, from 9% to 56% within 4 days. Similarly *Tulbaghia alliacea* seeds showed highest percentage germination after being shifted from a low temperature to a higher temperature (KULKARNI *et al*., 2005a). There was no increase in germination with the seeds shifted from 30 °C to 25 °C. This may be due to the detrimental effect of the high temperature on enzymes and seed reserves.

3.3.5. Phytochrome effects

Light plays a crucial role in germination, with light quality rather than quantity being more important (CASAL and SMITH, 1989). To investigate the possible involvement of phytochrome in seeds of *A. natalensis*, they were exposed to irradiation of different light qualities. There was no significant difference with seeds exposed to (R and Fr) tested irradiation and those in the dark. Similar results were observed by KULKARNI *et al*. (2006) for *Eucomis autumnalis* suggesting that the influence of phytochrome might be slight or absent. Under white light, 66% of *A. natalensis* seed germinated compared to the 18% control dark conditions (Table 3.1) and 80% seeds germinated under alternating light/dark (16 h photoperiod) regimes (Fig. 3.2). Similar results were reported for *Pachycereus pringlei*

(NOLASCO *et al.,* 1996). It is quite possible that the red/far-red light treatments were not applied at the best time. If one considers the imbibition curve (page 33) it can be seen that these seeds imbibe very slowly. Thus, it may have been better to have imbibed the seeds for 12 or 24h prior to red/far-red light treatments, at which time the seeds would have imbibed to about 20%. Perhaps the red-light treatment was unsuccessful because the seeds were treated when they were still quite dry.

Table 3.1: Effect of different light qualities on *A. natalensis* seed germination at 25 ℃.

Treatment	Germination (%)	MGT (days)
White light	66 ± 1 a	13 ± 0.0 d
Dark (control)	18 ± 1 b	19 ± 0.0 abc
R	18 ± 1 b	19 ± 1.0 abc
Fr-R	9 ± 1 b	15 ± 5.0 bc
R-Fr	14 ± 0 b	23 ± 3.0 a
Fr-R	15 ± 1 b	21 ± 1.0 ab
R-Fr-R	19 ± 0 b	19 ± 1.0 abc
Fr-R-Fr	13 ± 1 b	22 ± 3.0 ab

MGT= mean germination time. Mean values (± SE) in a column with different letters are significantly different ($p < 0.05$). R= red Fr= far-red

Seeds in their natural environment undergo shading by two means. One is when they are buried in soil, and the other is when they are under a canopy, in both cases this affects light quantity and quality (FRANKLAND, 1981). However, the results obtained in this investigation suggest that *A. natalensis* seeds will not germinate well under a canopy as they required full exposure to white light.

3.3.6. Effect of cold stratification

After 28 days of cold stratification, *A. natalensis* seeds exhibited significantly higher percentage germination (91%) than the non-stratified seeds and those that were subjected to shorter stratification periods (Fig. 3.4). Stratification treatment also decreased the MGT compared to the control.

Cold–wet stratification is regarded as the most efficient way to break dormancy in seeds of summer annual and most temperate perennials (BASKIN and BASKIN, 1998). However, a chilling requirement represents a mechanism to ensure that germination occurs in spring or early summer (PROBERT, 1992). In this study, the breaking of primary dormancy by low temperature stratification successfully enhanced seed germination of *A. natalensis* after 4 weeks of stratification, compared to the control. Similar studies were reported by NADJAFI *et al*. (2006) where the highest germination percentage of *Ferula gummosa* seeds was obtained by soaking seeds in water at 5 ℃.

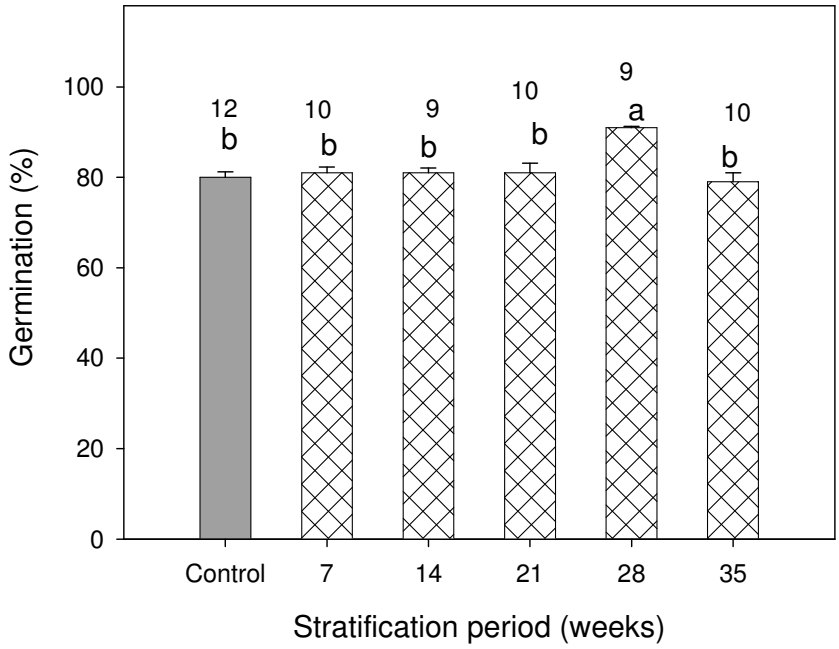

Figure 3.4: Effect of different cold stratification (5 ℃) periods on subsequent seed germination of *A. natalensis* incubated at a 16 h photoperiod. Bars (± SE) with different letters are significantly different ($p < 0.05$). The number above the letters represents MGT in days.

3.3.7. Effect of seed storage on seed germination

After 6 months of storage at room temperature, seeds of *A. natalensis* obtained highest germination percentage (99%) compared to the other storage conditions (5 ℃ and -20 ℃) (Table 3.2).

It has been reported that fresh seeds usually germinate under a specific narrow range of conditions. Such conditions gradually become wider as a result of seed storage (GUTTERMAN *et al*., 1998; OROZCO-SEGOVIA *et al*., 2000; QADERI *et al*., 2003). Dry storage frequently increase germination of stored seeds over that of fresh seeds in many species, including *Spergularia diandra* (GUTTERMAN, 2000), *Portulaca oleracea* (EL-KEBLAWY and AL-ANSARI, 2000), *Lonicera* spp. (HIDAYATI *et al*., 2002) and *Bromus tectorum* (ALLEN and MEYER, 2002). For *A. natalensis,* results are similar to these species where 6 months of storage yielded 99% germination.

Table 3.2: Effect of storage temperature and duration on viability of *A. natalensis* seeds.

	Storage temperature					
	25 ℃ (RT)		5 ℃		-20 ℃	
Month	Germination (%)	MGT (days)	Germination (%)	MGT (days)	Germination (%)	MGT (days)
1	84 ± 0.9 b	12 ± 0 a	76 ± 1.0 ab	13 ± 0 a	62 ± 2.1 a	12 ± 0 a
2	79 ± 1.1 b	11 ± 0 ab	79 ± 1.7 ab	12 ± 1 a	69 ± 1.4 a	10 ± 1 b
3	74 ±1.1 b	7 ± 0.2 ab	71 ± 0.8 ab	5 ± 0 b	73 ± 0.8 a	5 ± 0 c
4	75 ± 0.7 b	9 ± 0.2 ab	82 ± 2.3 a	5 ± 0 b	62 ± 1.5 a	5 ± 0 d
5	76 ± 0.7 b	7 ± 0.3 ab	64 ± 0.9 b	4 ± 1 b	67 ± 0.4 a	4 ± 0 d
6	99 ± 0.2 a	9 ± 0.2 ab	64 ± 1.0 b	5 ± 1 b	62 ± 1.1 a	4 ± 0 d

MGT = mean germination time. RT= Room temperature. Mean values (± SE) in a column with different letters are significantly different ($p < 0.05$). Seeds were incubated at 25 ºC under a 16 h photoperiod.

3.3.8. Effect of sowing depth

Sowing depth significantly affected seedling emergence (Table 3.3). At a depth of 0.5 cm, the highest germination percentage was recorded (35%), as sowing depth increased seedling emergence decreased. When deeper in the soil there is an oxygen supply shortage, which could be the reason for the difficulty of seedling emergence (BOYD and VAN ACKER, 2004). However, when seeds are placed on the soil surface, there is physical damage to embryos and roots from strong sunlight that causes greater mortality of seedlings (YANG *et al*., 2008).

Table 3.3: Effect of sowing depth on seed germination of *A. natalensis.*

Sowing depth (cm)	Germination (%)	MGT (days)
0.5	35 ± 1 a	10 ± 1.0 a
1.0	33 ± 1 a	10 ± 1.0 a
1.5	28 ± 0 a	10 ± 2.0 a
2.0	15 ± 1 b	6 ± 2.0 a
2.5	5 ± 0 b	6 ± 3.0 a

MGT = mean germination time. Mean values (± SE) in a column with different letters are significantly different ($p < 0.05$).

3.3.9. Effect of smoke solution

Treating *A. natalensis* seeds with both smoke-water and butenolide stimulated seed germination. Smoke-water (1:500 v/v) and butenolide (10^{-8} M) improved seed germination (83%) under alternating light conditions (16 h photoperiod) compared to the control (Table 3.4). Under constant dark, butenolide (10^{-9} M) showed slight improvement in percentage germination of *A. natalensis* seeds. However, smoke-water and butenolide had no significant effect when seeds were germinated under constant light (Table 3.4).

For many years smoke-water has been known to stimulate germination of indigenous plants. According to LIGHT and VAN STADEN (2004) the stimulatory effect of smoke is now well recognized and shows great potential in seed biology.

Table 3.4: Influence of smoke-water (1:500 v/v) and butenolide on seed germination of *A. natalensis* under different light conditions at 25 °C.

		Germination (%)	
Condition	Treatment	*A. natalensis*	MGT
Alternating light (16:8 light/dark)	Control	71 ± 1.0 a	13 ± 0.6 b
	Smoke-water	83 ± 1.0 a	13 ± 0.4 a
	Butenolide 10^{-7} M	61 ± 1.0 a	10 ± 0 b
	Butenolide 10^{-8} M	83 ± 1.2 a	14 ± 0.9 a
	Butenolide 10^{-9} M	65 ± 0 a	11 ± 0 b
Constant light	Control	76 ± 2.0 a	13 ± 1.0 a
	Smoke-water	68 ± 1.0 a	13 ± 1.0 a
	Butenolide 10^{-7} M	75 ± 2.0 a	12 ± 0 ab
	Butenolide 10^{-8} M	70 ± 0 a	11 ± 0 c
	Butenolide 10^{-9} M	76 ± 1.0 a	12 ± 0 ab
Constant dark	Control	54 ± 0 ab	15 ± 1.0 b
	Smoke-water	41 ± 0 bc	15 ± 1.0 b
	Butenolide 10^{-7} M	10 ± 0 d	19 ± 1.0 a
	Butenolide 10^{-8} M	24 ± 1.0 cd	15 ± 0 b
	Butenolide 10^{-9} M	69 ± 2.0 a	16 ± 0 ab

MGT = mean germination time. Mean values (± SE) in a column with different letter(s) are significantly different ($p < 0.05$).

3.3.10. Effect of smoke solution and chemical substances

Seeds of *A. natalensis* treated with GA_3 (10^{-8} M) solution exhibited significantly higher percentage germination (98%) compared with the other treatments and the control (Table 3.5).

Table 3.5: Effect of smoke solutions and chemical substance on seed germination of *A. natalensis* at 25 ℃ under a 16 h photoperiod.

Treatment	Germination (%)	MGT (days)
Control	71.0 ± 1.0 d	12.5 ± 0.6 b
GA_3 (10^{-6} M)	91.0 ± 1.2 ab	12.5 ± 0.2 b
GA_3 (10^{-7} M)	89.0 ± 1.0 ab	12.0 ± 0.4 b
GA_3 (10^{-8} M)	98.0 ± 0.8 a	13.0 ± 0.4 b
Kinetin (10^{-6} M)	64.0 ± 1.0 d	12.5 ± 0.2 b
Kinetin (10^{-7} M)	76.0 ± 1.0 cd	12.2 ± 0.4 b
Kinetin (10^{-8} M)	75.0 ± 2.0 cd	12.2 ± 0.4 b
KNO_3(10^{-6} M)	67.0 ± 1.0 d	15.0 ± 1.0 ab
KNO_3(10^{-7} M)	63.0 ± 1.0 d	18.0 ± 1.0 a
KNO_3(10^{-8} M)	78.0 ± 2.0 cd	16.0 ± 1.0 b
Butenolide (10^{-7} M)	61.0 ± 0.0 d	10.0 ± 0 b
Butenolide (10^{-8} M)	82.5 ± 1.0 bc	13.7 ± 0.9 a
Butenolide (10^{-9} M)	65.0 ± 0.0 d	11.0 ± 0 b
Smoke-water (1:500 v/v)	82.5 ± 1..2 bc	12.7 ± 0.4 a

MGT= mean germination time. Mean values (± SE) in a column with different letters are significantly different ($p<0.05$).

In this study, application of all three concentrations of GA_3 exhibited the best percentage germination of *A. natalensis* seeds (Table 3.5). This indicates that GA_3 activates the physiological metabolism of the seed which can release dormancy or enhance germination of high-altitude species like *A. natalensis.* In a recent study, moist-chilling and GA_3 application enhanced percentage germination of three *Pedicularis* species which are mainly distributed in high-altitudes (REN and GUAN, 2008).

3.3.11. Effect of smoke solutions at different temperature and light conditions on seed germination

Smoke-water and butenolide significantly increased the germination percentage of *A. natalensis* seeds under 10, 15, 20 and 25 ℃ within a shorter period of time compared to the control. However, smoke-water (1:500 v/v) at 15 ℃ showed the highest germination percentage (88%) (Fig. 3.5 A). The stimulating effects of smoke-water and butenolide under low temperatures is of great significance as these results suggest that smoke-water and butenolide treatments can be useful in achieving maximum percentage germination of *A. natalensis* seeds even at low temperatures.

Under dark condition at 10 ℃ and 30 ℃, seeds of *A. natalensis* failed to germinate. High temperatures may prevent germination because metabolic rates are reduced due to protein denaturation which affects permeability of membranes while at low temperatures, reduced enzyme activity results in low metabolism (DELACHIAVE and DE PINHO, 2003). There was a slight increase in germination under light conditions (Fig. 3.5 B).

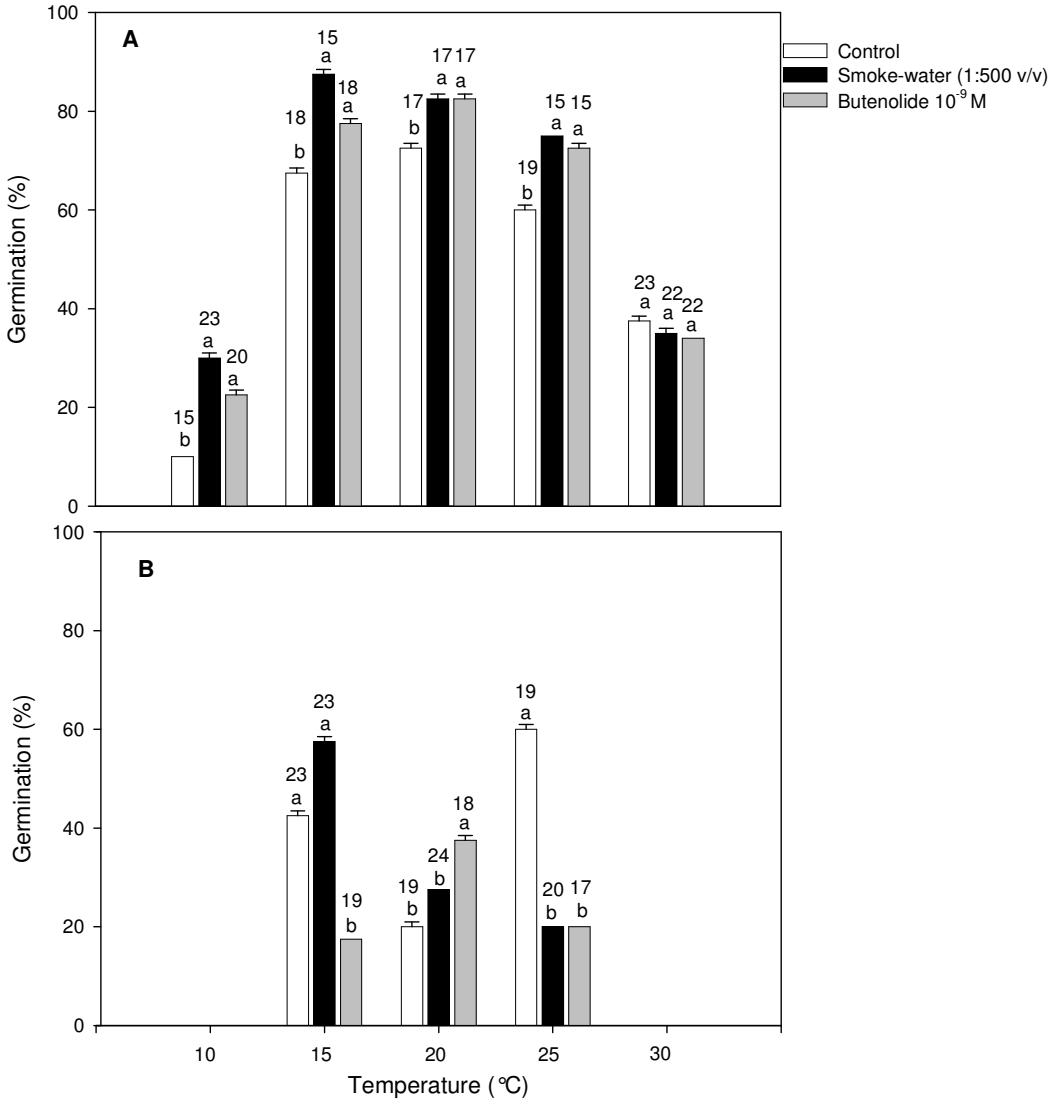

Figure 3.5: Effect of smoke-water (1:500 v/v) and butenolide 10^{-9} M on seed germination of *A. natalensis* under (A) light conditions with a 16 h photoperiod and (B) in the dark Bars (± SE) with different letters are significantly different ($p < 0.05$). The number above the letters represents MGT in days.

3.3.12. Effects on seedling vigour

Smoke-water-treated seeds of *A. natalensis* at 15, 20 and 25 ℃ and butenolide at 25 ℃ showed significantly greater seedling vigour indices than the untreated seeds. However, no seedlings developed at 10 and 30 ℃. At 25 ℃ butenolide and smoke promoted the growth of *A. natalensis*, whilst smoke-water and butenolide did not have a promotive effect on growth of *A. natalensis* below 20 ℃ (Table 3.6).

Table 3.6: Effect of smoke-water (1:500 v/v) and butenolide (10^{-8} M) solutions on seedling growth of *A. natalensis* at different temperatures under a 16 h photoperiod.

Temperature	Treatment	Seedling length (mm)	Seedling vigour index
15 ℃	Control	30.5 ± 1.5 b	2058 ± 105 b
	Smoke-water	39.8 ± 2.3 a	3482 ± 209 a
	Butenolide	23.7 ± 1.7 c	1836 ± 135 b
20 ℃	Control	29.3 ± 1.2 a	2124 ± 91 b
	Smoke-water	29.3 ± 2.2 a	2417 ± 120 a
	Butenolide	26.2 ± 1.5 a	2161 ± 130 b
25 ℃	Control	33.9 ± 2.6 a	2406 ± 159 b
	Smoke-water	36.9 ± 2.5 a	3044 ± 127 a
	Butenolide	37.4 ± 3.2 a	3085 ± 136 a

MGT = mean germination time. Mean value of each temperature in a row (± SE) with different letters are significantly different ($p < 0.05$).

Recent studies have shown that the effects of smoke extend beyond post-germination events resulting in the stimulation of seedling vigour (BAXTER and VAN STADEN, 1994; SPARG *et al*., 2005b KULKARNI *et al*., 2007). Not only was there an improvement in percentage germination of *A. natalensis* seeds, but the seedling vigour was also significantly enhanced by smoke-water treatment at temperatures of 15, 20 and 25 ℃ (Table 3.6). Similar effects of smoke-water and butenolide have been reported for tomato seedlings (JAIN *et al*., 2006). However, butenolide was only effective at 25 ℃. This is probably because butenolide may

be exerting its effects through synergistic action with other compounds present in smoke-water.

3.4 Conclusions

The seeds of *A. natalensis* require alternating light conditions for optimum germination. The most favourable temperature for germination of *A. natalensis* was 25 °C. Cold stratification (5 °C) for 28 days improved germination of *A. natalensis* seeds. Seed of *A. natalensis* can be germinated under white light. Thus the species is positively photoblastic. Sowing depth results revealed that the seeds of *A. natalensis* need to be sown near the soil surface. Smoke-water was beneficial in enhancing percentage germination of *A. natalensis* seeds, while GA_3 (10^{-8} M) was the best treatment in promoting maximum germination. The seeds of *A. natalensis* can be treated with smoke-water to improve both germination and seedling vigour.

CHAPTER 4. ANTIMICROBIAL, ANTI-INFLAMMATORY AND GENOTOXICITY ACTIVITY OF *Alepidea amatymbica* AND *Alepidea natalensis*

4.1. Introduction

Pharmacological studies have been carried out on a number of commonly used medicinal plants to establish a scientific rationale for their usage in traditional medicine. There is also a need to determine whether plants that are used in traditional medicine are toxic to humans (SPARG, 2003).

The people of South Africa have a long history of traditional plant usage for the treatment of various diseases and ailments (CUNNINGHAM, 1993; VAN WYK *et al*., 1997, LIGHT *et al*., 2005; KAMBIZI and AFOLAYAN, 2008). Because of the widespread use of medicinal plants by indigenous people, the search for biologically active agents based on traditionally used plants is still flourishing. Medicinal plants have the potential to provide pharmacologically active natural products (CRAGG *et al*., 1997; ELDEEN and VAN STADEN, 2007).

4.1.1. Objectives of this study

To investigate the antibacterial, antifungal, anti-inflammatory and genotoxic effects of the two *Alepidea* species (*A. natalensis* and *A. amatymbica*).

4.2. Sample collection and preparation

4.2.1. Plant material

Plant material was collected in March, 2007 from Mount Gilboa (29°16.766´S, 30°17.627´E) near Pietermaritzburg, KwaZulu-Natal, South Africa Voucher specimens *A. natalensis* Wood & Evans. [Mulaudzi 01 (NU)] and *A. amatymbica* Eckl. & Zeyh.[Mulaudzi 12 (NU)] were deposited at the University of KwaZulu-

Natal Herbarium, Pietermaritzburg. Plant material was separated into leaves and rhizomes and dried at 50 ℃ for 3 days, ground and stored in airtight containers at 10℃ in the dark.

4.2.2. Preparation of plant extracts

Dried, ground plant material (5 g) was extracted sequentially with 100 ml of petroleum ether (PE), dichloromethane (DCM), 80% ethanol (EtOH) and water with sonication for 1 h each. The temperature was kept low by adding ice to the sonication bath. The extracts were filtered through Whatman No. 1 filter paper and concentrated under vacuum using a rotary evaporator. The concentrates were dried at room temperature under a stream of air and then kept at 10 ℃ in the dark until required.

4.3. Antibacterial screening

4.3.1. Introduction

Bacteria are amongst the most abundant organisms on earth and are able to adapt to almost any living condition. They are both useful and harmful to humans. Bacterial infections are very common and frequently cause death. Antibacterial drugs remain the most important therapeutic method of dealing with bacterial infections (PAGE *et al*., 1997).

There are two major classes of bacteria, Gram-positive and Gram-negative, determined by a staining technique. Bacteria cells are stained with gentian violet and then treated with Gram's solution. After being decolourized with alcohol and treated with safranine and washed in water, those that retain the gentian violet are Gram-positive and those that do not retain it are Gram-negative (SALTON and KIM, 1996).

Some of the common bacteria that are known to cause infections are *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumonia.*

Bacillus subtilis is a Gram-positive aerobic rod-shaped spore-producing bacterium. *Bacillus subtilis* often occurred in chainlike formations, found primarily in soil and causes food poisoning (RYAN and RAY, 2004). *Staphylococcus aureus* is a spherical Gram-positive parasitic bacterium that causes illnesses ranging from minor skin infections and abscesses, to life-threatening diseases such as pneumonia, meningitis and septicaemia (LOWY, 1998). *Escherichia coli* is a Gram-negative bacterium normally present in the intestinal tract of humans and other animals. *Escherichia. coli* can sometimes be pathogenic thus posing a threat to food safety, causing diarrhoea, wound and urinary infections (SLEIGH and TIMBURY, 1988). *Klebsiella pneumonia* is a genus of non-motile rod-shaped Gram-negative enterobacteria which cause respiratory and other infections (RYAN and RAY, 2004).

Development of antimicrobial agents for clinical use has brought unquestionable benefit to individuals and society (LERNER, 1998). Infectious diseases that were formerly often incurable can now be cured. However, mankind is now confronted with new and re-emerging infections for which no effective treatments are available (CRAGG *et al*., 1997). In contrast to other types of medication, antibiotics ultimately lose their effectiveness as resistant strains of bacteria develop (LERNER, 1998). An example is the Gram-positive, methicillin-resistant *Staphylococcus aureus*. It was reported in some hospitals that more than 40% of *S. aureus* strains are now resistant to methicillin (LESSE, 1995). However, natural products could play a crucial role in meeting the demand for new drugs against infectious diseases (CRAGG and NEWMAN, 2001).

Various methods of screening for antibacterial activity have been evaluated. These could be classified into three groups, disc-diffusion, microdilution and bioautographic assays (RIOS *et al*., 1988). The disc-diffusion assay is one of the most common forms of testing for antibacterial activity, and does not require homogenous dispersion of the extract and also allow several plant extracts to be screened against various bacteria at the same time. Microdilution techniques generally require a homogeneous dispersion of the sample in water and are used to determine more precisely the antibacterial activity of the extracts. They are often used to determine the minimum inhibitory concentration (MIC) or minimum

bactericidal concentration (MBC) of test extracts for a given microorganism (PAGE *et al*., 1997). The bioautographic assays allows for rapid detection of bioactive constituents of plant extracts during bioassay-guided fractionation of antibacterial compounds (RASOANAIVO and RATSIMAMANGA-URVERG, 1993).

4.3.2. Materials and Methods

4.3.2.1. Extract preparation

Extracts of the rhizomes of *A. amatymbica*, leaves and rhizomes of *A. natalensis* were prepared as described in section 4.2.2. The residues from the plant extracts were redissolved to a concentration of 50 mg/ml in water (for aqueous extracts) or 80% ethanol (for DCM, PE and EtOH extracts).

4.3.2.2. The microdilution bioassay

Minimum inhibitory concentration (MIC) for antibacterial activity was determined using the microdilution bioassay in a 96-well microplates (ELOFF, 1998). One hundred microlitres of each redissolved sample (50 mg/ml) in 80% ethanol were two-fold serially diluted with sterile distilled water, in duplicate down a 96-well microplate for each of the four bacteria employed. A similar two-fold serial dilution of neomycin (0.1 mg/ml, Sigma) was used as a positive control against each bacterium. Water and bacteria-free MH broth were included as negative controls. Overnight Mueller-Hinton (MH) broth cultures (incubated at 37 ℃ in a water bath with shaking) of four bacterial strains: two Gram-positive (*Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 12600) and two Gram-negative (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883) were diluted with sterile MH broth (1 ml bacteria/50 ml MH broth). One hundred microlitres of each bacterial culture were added to each well. The plates were covered with parafilm and incubated overnight at 37 ℃. Bacterial growth was tested by adding 50 µl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) to each well and the plates incubated at 37 ℃ for 1 h. Bacterial growth in the wells was indicated by a red-pink colour, whereas clear wells indicated inhibition of growth by

the test sample. MIC values were recorded as the lowest concentration of extract showing a clear well. Each assay was repeated twice with two replicates.

4.3.3. Results and Discussion

Results for antibacterial activity are presented in Table 4.1. An extract having an antibacterial MIC less than 1 mg/ml is considered to have good antibacterial activity (ALIGIANNIS *et al*., 2001). Poor inhibitory activity was detected for EtOH extract of *A. natalensis* leaf as well as water extract of *A. amatymbica* rhizome. Good inhibition was observed for PE and DCM extracts of *A. natalensis* leaf while *A. amatymbica* rhizome exhibited the best activity against *Bacillus subtilis* (MIC value of 0.39 mg/ml). *A. natalensis* rhizomes showed good inhibitory activity for water extract for both Gram-positive and Gram-negative bacteria.

The results suggests that the water extracts of *A. natalensis* rhizome contains compounds with high activity against *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* despite reports that water extracts often do not have good activity (LUSEBA *et al*., 2007). However, this was not the case for the leaf extracts of *A. natalensis* as well as the rhizome water extracts of *A. amatymbica* which showed low activity against all the bacteria strains used (Table 4.1). Traditionally, water and not organic solvents are used to make decoctions. The *A. amatymbica* rhizome decoction is a popular remedy against colds and chest complaints (DE CASTRO and VAN WYK, 1994). The activity observed in the water extracts of *A. natalensis* in our bioassays suggests that it could substitute for *A. amatymbica* for use against bacterial infections. The rhizome of *A. amatymbica* contains high concentrations of diterpenoids of the kaurene type, the major compounds being dehydrokaurenoic acid and kaurenoic acid. The anti-microbial activity of these two species may be due to the diterpenoids, although the compounds have not been tested individually (HUTCHINGS, 1989a; VAN WYK *et al*., 1997; VAN WYK *et al*., 2008).

Table 4.1: Antibacterial minimal inhibitory concentration (MIC) of extracts from two *Alepedia* species.

Extract	MIC (mg/ml)											
	A. natalensis leaf				*A. natalensis* rhizome				*A. amatymbica* rhizome			
	B.s.	*E.c.*	*K.p.*	*S.a.*	*B.s.*	*E.c.*	*K.p.*	*S.a.*	*B.s.*	*E.c.*	*K.p.*	*S.a.*
PE	**0.78***	1.56	1.56	3.13	3.13	3.13	3.13	3.13	**0.39**	3.13	3.13	3.13
DCM	**0.78**	3.13	1.56	**0.78**	1.56	3.13	1.56	3.13	**0.39**	3.13	3.13	1.56
EtOH	3.13	3.13	1.56	3.13	3.13	3.13	**0.78**	3.13	1.56	**0.78**	**0.78**	1.56
Water	12.5	12.5	12.5	12.5	**0.78**	**0.78**	**0.78**	**0.78**	1.56	1.56	1.56	1.56

B.s.= Bacillus subtilis; *E.c.= Escherichia coli*; *K.p.= Klebsiella pneumoniae*; *S.a.= Staphylococcus aureus*

*Values boldly-written are considered very active (< 1 mg/ml). The MIC values (µg/ml) for Neomycin (positive control) were: *B. subtilis* = 1.6 $\times 10^{-3}$; *E. coli* = 0.8×10^{-3}; *S. aureus* = 0.8×10^{-3}; *K. pneumoniae* = 1.6×10^{-3}.

4.4. Antifungal activity

4.4.1. Introduction

Fungi form a large group of heterotrophic organisms which exist as saprophytes, parasites or commensals in the soil or on decaying plant material (GREENWOOD *et al*., 1992). There are three types of fungi; moulds, yeasts and mushrooms, some fungi are useful whereas others may be toxic and harmful.

Fungi play an important role in the degradation of organic compounds. They are being used as sources of antibiotics in medicine such as pencillin and various enzymes such as pectinase, cellulase and protease (SLEIGH and TIMBURY, 1988).

Fungal infection creates an inflammatory condition characterised by an irritating patch of itchy skin (SLEIGH and TIMBURY, 1988). Common infections are vaginal and oral thrush, which can be life threatening.

Candida albicans is one of the parasitic fungi that can infect the mouth, skin, intestines or the vagina and is among the many organisms which live in the human population with no harmful effects. However, overgrowth results in *candidiasis,* known as thrush (RYAN and RAY, 2004).

Rapid increases in disease associated with fungal infection such as *candidiasis* are related with the increased rate of HIV infection in many communities (McNEIL *et al*., 2001; WHITE *et al*., 1998).

Amphotericin B (AmpB) and the azole group of antifungal agents are widely used in the treatment of fungal infections. Unfortunately, the widespread and incorrect use of these antifungal agents has led to the emergence of drug resistance in several common pathogenic fungi (GRAYBILL, 1996). Due to this emergence of antibiotic resistance by human pathogenic fungi, it is important to develop new antifungal agents. The field of ethnobotanical research has expanded greatly in

recent years. Plants may yield valuable antifungal compounds (MASOKO *et al*., 2007).

Amphotericin B is the most effective drug for treating most life-threatening fungal infections (SAU *et al*., 2003). However, therapeutic use of AmpB is limited by its toxicity to patients (MANDELL *et al*., 2000). According to SAU *et al*. (2003) AmpB toxicity has provided the thrust for the development of multiple, alternative antifungal drugs such as lipid formulations of AmpB, the azoles and the echinocandins. Regardless of alternating therapies it is still widely administered because it rapidly acts as a fungicide, has a broad spectrum of antifungal activity and it rarely induces resistance. The resistance of *Candida* species to azole compounds has been investigated over the past few years. As a consequence of the AIDS epidemic during the past decade there was a striking increase in mucosal infections caused by *Candida* species which are associated with a worrying emergence of resistance to azoles. The resistance to AmpB has emerged in parallel with the increase in the number of invasive infections due to the so-called emerging fungi. The emerging fungi are yeasts such as *Trichosporon beigelii*, *C. lusitaniae* or *C. guillermondii*. Many of these fungi show primary resistance to AmpB, and may cause invasive infections, usually associated with a high mortality (TRITZ and WOODS, 1993).

There is still a high death rate associated with some invasive fungal infections, especially those produced by filamentous fungi. HOSTETTMANN *et al.* (2000) stressed the importance of investigating plants for new antifungal agents.

4.4.2. Materials and Methods

A modification of the National Committee for Clinical Laboratory Standards (NCCLS) proposed method (M27-P), the broth dilution test, was used to assess the antifungal activity of plant extracts (ESPINEL-INGROFF and PFALLER, 1995). The water extract residues were redissolved in water and the organic solvent extract residues in dimethyl sulfoxide (DMSO). All extracts were diluted to a concentration of 50 mg/ml. Each extract was tested against a standard strain of *Candida albicans* (ATCC 10231).

Cultures of *C. albicans* were inoculated in yeast mould (YM) broth (Sigma) and incubated for 24 h at 37 ℃ in a water-bath on an orbital shaker. Sterile saline (4 µl) was added to approximately 400 µl of 24 h old *C. albicans* cultures. The absorbance was read at 530 nm and adjusted with sterile saline to match that of a 0.5 McFarland standard solution (HARRIGAN, 1998). From the prepared yeast stock culture, a 1: 1000 dilution with broth was prepared (10 µl yeast stock culture: 10 ml broth).

Fifty microlitres of organic solvent extracts were serially diluted 2-fold with 175 µl sterile YM broth. In the case of aqueous extracts, 100 µl of extract were added to 100 µl of broth and serially diluted. A similar 2-fold serial dilution of AmpB was used as a positive control. Broth, fungal strain with no extract, water and DMSO were included as negative controls. To each of the wells containing the test and control solutions, 100 µl of the yeast cultures were added and incubated for 24 h at 37 ℃. To indicate fungal growth, 50 µl of 0.2 mg/ml p-iodonitrotetrazolium chloride (Sigma) (INT) were added to each well, and the plates were incubated for a further 24 h. The wells which displayed no change in colour indicated antifungal activity. The minimum inhibitory concentration (MIC) was taken as the lowest concentration of plant extract to inhibit growth of the tested fungus after 48 h respectively. After 72 h YM broth 50 µl was added to determine whether the inhibition was fungicidal or fungistatic. The minimum fungicidal concentration (MFC) was taken as the last clear well observed after addition of YM broth and further incubation at 37 ℃ for 24 h. The assay was repeated three times for each extract.

4.4.3. Results and Discussion

The antifungal assay results as MIC and minimum fungicidal concentrations (MFC) are presented in Table 4.2. *A. natalensis* leaf extracts exhibited good antifungal activity with MIC values ranging from 0.2 to 0.78 mg/ml for all extracts. It is important to determine whether the active extracts were fungistatic or fungicidal. This was done by adding broth to all clear wells on the microtitre plate and incubating the plate for a further 24 h. The last clear well was then recorded as the

MFC. It was noted that the DCM extract of *A. natalensis* leaf, was fungicidal at lower concentration (MIC=MFC) as there was no change in the values of the last clear well after further addition of broth and 24 h incubation.

Table 4.2: Antifungal activity (MIC and MFC) of different extracts of two *Alepidea* species against *Candida albicans*.

Plant part	Extract	MIC (mg/ml)	MFC (mg/ml)
A. natalensis	PE	**0.78***	1.56
leaf	DCM	**0.20**	**0.20**
	EtOH	**0.78**	3.13
	Water	**0.78**	6.25
A. natalensis	PE	6.25	6.25
rhizome	DCM	3.13	6.25
	EtOH	6.25	6.25
	Water	1.56	1.56
A. amatymbica	PE	6.25	6.25
rhizome	DCM	1.56	1.56
	EtOH	6.25	6.25
	Water	**0.20**	1.56
Amphotericin B (µg/ml)		9.77×10^{-3}	7.81×10^{-2}

*Values boldly-written are considered very active (< 1 mg/ml).

The low activity values in some of the extracts tested in the present assays could be due to the impure form and/or low concentration of the active compound(s) in the crude extracts (RABE and VAN STADEN, 1997). *A. natalensis* and *A. amatymbica* rhizome extracts showed poor activity against *C. albicans*. However, some of the plant extracts, as with some drugs, may be more potent *in vivo* due to metabolic transformation of some components into highly active intermediates or may interact with the immune system (GARCIA *et al*., 2003; NGEMENYA *et al*., 2006).

4.5. Anti-inflammatory activity

4.5.1. Introduction

Inflammation is the complex biological response of vascular tissue to harmful stimuli of damaged cells. It is classified as acute or chronic, and described as the basis of many human diseases (GUO *et al.*, 2008). There are many inflammatory mediators that participate in the regulation of inflammation. The mediator responses include vascular amines and metabolites of arachidonic acid (prostaglandins, leukotrienes and lipoxins) (WEI *et al.*, 2004).

Inflammation responses are triggered by the biosynthesis from arachidonic acid of a group of compounds known as prostaglandins. These reactions are catalysed by cyclooxygenase (COX) enzymes (SALMON and HIGGS, 1994). Prostaglandins are used to regulate the inflammatory response. They are potent substances that act like hormones and are found in many bodily tissues, produced in response to trauma and may affect blood pressure and metabolism as well as smooth muscle activity.

According to NASIRI *et al*. (1993), drugs for inflammation target the COX enzyme, preventing the formation of prostaglandins. Such drugs are known as non-steroidal anti-inflammatory drugs (NSAIDs) (RANG and DALE, 1987). Aspirin is an NSAID (COX and BLACK, 1994). NSAIDs were widely used in the last century for the treatment of various inflammatory diseases. However, their mechanism of action was not fully appreciated until 1971 when their molecular target, the COX enzyme was identified (VANE and BOTTING, 1987). VANE and BOTTING (1996) found that this enzyme consists of two isozymes COX-1 and COX-2. COX enzymes are involved in the synthesis of prostaglandins that cause inflammation pain and fever (LI *et al.,* 2006). It has been suggested that constitutive COX-1 is involved in homoeostatic processes whereas COX-2 plays a major part in the inflammatory reactions (LI *et al.,* 2006).

4.5.2. Materials and Methods

4.5.2.1. COX-1 bioassay

The COX-1 bioassay was performed as described by ELDEEN and VAN STADEN (2008). The COX-1 enzyme isolated from ram seminal vesicles was obtained from Sigma-Aldrich. A stock solution of COX-1 enzyme was stored at -70 ℃ until use. Ten microlitre of the enzyme containing 3 units were prepared and stored at -70 ºC until use. The enzyme was activated with 1250 µl of co-factor solution (0.3 mg/ml L-adrenaline and 0.3 mg/ml reduced glutathione in 0.1 M Tris buffer, at pH 8.0) on ice for 5 min. Sixty microlitres of the enzyme solution were added to each sample solution (2.5 µl plant extract and 17.5 µl water) and incubated at room temperature for 5 min. The extracts were tested at a concentration of 10 mg/ml (resuspended in 80% ethanol) giving a final concentration of 250 µg/ml per test solution. Indomethacin at 5 µM, background (the enzyme was inactivated with HCl before addition of ^{14}C-arachidonic acid) and solvent blank were used as controls.

The reaction was started by adding 20 µl ^{14}C-arachidonic acid (16 Ci/mol, 30 µM) to each sample. Samples were incubated in a water bath at 37 ℃ for 10 min and afterwards the reaction was stopped by adding 10 µl 2N HCl. Four microlitres (0.2 mg/ml) of unlabeled prostaglandins (PGE_2: $PGF_{2\alpha}$ 1:1) (Sigma-Aldrich) were added to each sample as a carrier solution. Silica columns were packed with silica gel (silica gel 60, 0.063-0.200 mm, Merck) to a height of 3 cm in Pasteur pipettes. The test solution was applied to the column with 1 ml of eluent 1 [hexane: 1, 4-dioxan: glacial acetic acid (70:30:0.2)]. This was done to separate the prostaglandins and unmetabolized arachidonic acid. The arachidonic acid was eluted first with 4 ml eluent 1 and the prostaglandin products were eluted with 3 ml of eluent 2 [ethyl acetate: methanol (85:15)] and collected in scintillation vials. To each vial 4 ml of scintillation fluid were added and the disintegration per minute (DPM) of radioactive material was counted using a scintillation counter (Beckman LS 6000LL scintillation counter). For each assay, a duplicate set of samples were tested and the assay was repeated 3 times. Inhibition percentage was calculated using the equation below:

COX inhibition (%) = $\left[1 - \left(\frac{\text{DPMsample} - \text{DPMbackground}}{\text{DPMblank} - \text{DPMbackground}}\right)\right] \times 100$

4.5.2.2. COX-2 bioassay

The COX-2 activity was assessed using a method described by NOREEN *et al*. (1998), with slight modifications (ZSCHOCKE and VAN STADEN, 2000). Human recombinant COX-2 enzyme containing a six histidine sequence near the N-terminus isolated from a Baculovirus over expression system in S*f* 21 cells was used (Sigma-Aldrich). Ten microlitre of the enzyme containing 3 units were prepared and stored at -70 °C until use. The enzyme was activated with 50 µl co-factor solution (0.6 mg/ml L-adrenaline, 0.3 mg/ml reduced glutathione and 1 µM hematin in 0.1 M Tris buffer, pH 8.0) on ice for 5 min. Sixty microlitre of the enzyme solution were added to each sample solution (2.5 µl plant extract and 17.5 µl water) and incubated at room temperature for 5 min. The extracts were tested at a concentration of 10 mg/ml (resuspended in 80% ethanol) giving a final concentration of 250 µg/ml per test solution. Indomethacin at 200 µM, background and solvent blank were used as controls. The reaction was started by adding 20 µl ^{14}C-arachidonic acid (16 Ci/mol, 30 µM) to each sample. Samples were incubated in a water bath at 37 °C for 10 min and afterwards the reaction was stopped by adding 10 µl 2N HCl. The separation of prostaglandins and unmetabolized arachidonic acid was done as described for the COX-1 assay. For each assay, a duplicate set of samples were tested and the assay was repeated twice.

Four controls were run. Two were background in which the enzyme was inactivated with HCl before the addition of ^{14}C-arachidonic acid acid, and two were solvent blanks. Indomethacin was included as a standard. Percentage inhibition of the tested compound was calculated by comparing the amount of radioactivity present in the sample to that in the blank solvent as calculated in Section 4.5.2.1.

4.5.2.3. Statistical analysis

Percentage COX Inhibition was expressed as mean ± SE of three independent experiments. The software used was the Graph Pad Prism Version 4.00 statistical software program for Windows (GraphPad Software Inc.).

4.5.3. Results and Discussion

The prostaglandin inhibitory activity of the extracts in the in the COX-1 and COX-2 assays in Figure 4.1. Activity above 70% is considered to be relatively good for the concentration of the plant extract tested (TAYLOR and VAN STADEN, 2001). All the PE and DCM extracts had high COX-1 activity while the EtOH extracts for both *A. natalensis* and *A. amatymbica* had inhibition less than 40%. There were no significant differences in the percentage inhibition of COX-1 exhibited by the PE and DCM extracts of both *A. natalensis* and *A. amatymbica*. All the PE extracts showed higher COX-2 activity than COX-1. There were no significant differences in the activity exhibited by DCM extracts in COX-1 and COX-2. PE and DCM extracts both yielded percentage inhibition above 70% for both COX-1 and COX-2.

High COX-1 activity is undesirable as it has been reported to cause damage to the gastrointestinal tract (LUSEBA *et al*., 2007). EtOH extracts showed higher COX-2 activity for *A. natalensis* and *A. amatymbica* rhizomes than COX-1 activity. Water extracts exhibited moderate activity (between 40% and 60%) for *A. natalensis* leaf and *A. amatymbica* rhizome for COX-1 inhibition, while *A. natalensis* rhizome showed very low activity (<10%). All the water extracts showed very low activity (<20%) for COX-2 inhibition. It has often been reported that activity in water extracts is not detected or yields false positives possibly due to protein binding capability of phenolics (LUSEBA *et al*., 2007). However, some phenolic compounds such as proanthocyanidins have antioxidant effects and have been used to treat inflammatory diseases and wound healing (NDHLALA *et al*., 2008).

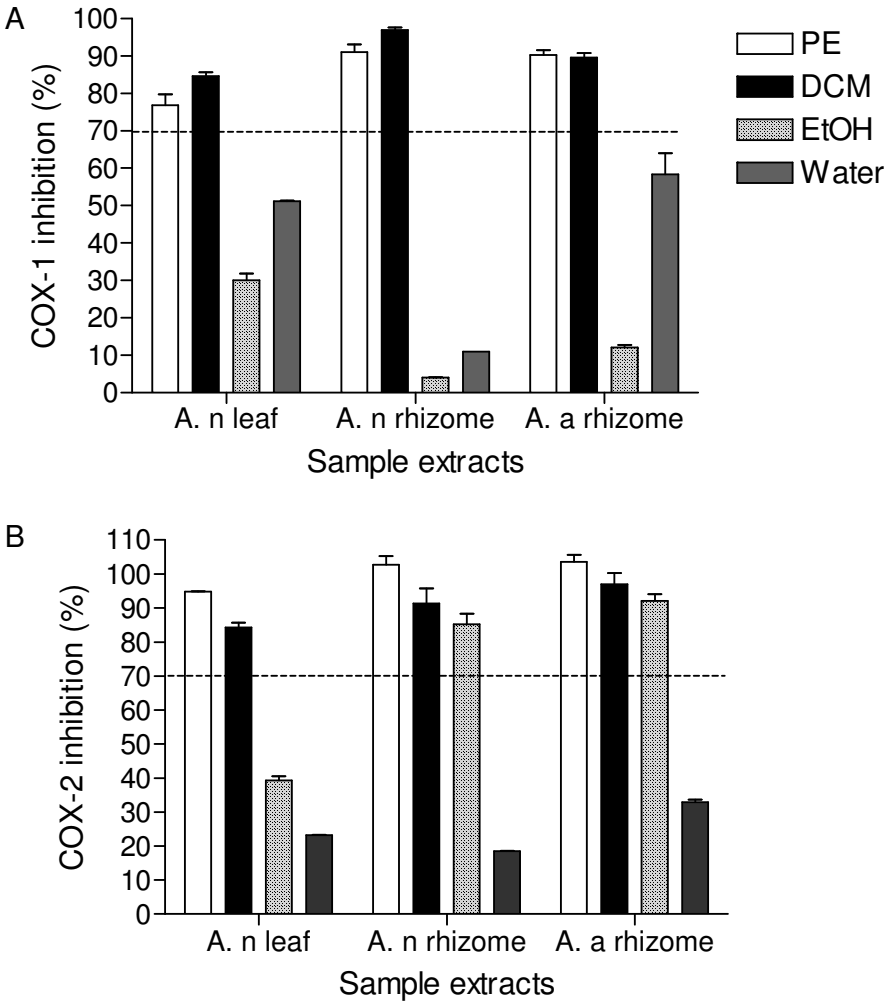

Figure 4.1: Percentage inhibition of (A) COX-1 and (B) COX-2 enzymes by *A. natalensis* leaf (A. n leaf), *A. natalensis* rhizome (A. n rhizome) and *A. amatymbica* rhizome (A. a rhizome). Indomethacin control for COX-1 (5 µM) and COX-2 (200 µM) gave 60% and 64% inhibition, respectively. All extracts with inhibition above 70% were considered to be active. All the extracts were tested at 250 µg/ml.

4.6. Genotoxicity activity

4.6.1. Introduction

Medicinal plants have been used since antiquity in the treatment of human diseases. Homemade herbal preparations are the most used medicines throughout the world. This is because they are believed to possess

pharmacological properties. However, they may also cause damage such as deoxyribonucleic acid (DNA) mutations. Therefore it is important to evaluate the mutagenic potential of plant extracts because of their long term usage as this has a major impact on the health of a large number of people (MARQUES et al., 2003).

Research has shown some substances present in medicinal plants are potentially toxic and carcinogenic (DE SÁ FERREIRA and FERRÃO VARGAS, 1999). Investigation of traditionally used medicinal plants is thus valuable at two levels, firstly as a source of potential chemotherapeutic drugs and secondly, as a measure of safety for continued use (VERSCHAEVE et al., 2004).

The adverse effects of widely used plant material are not well documented in the literature. Based on their long-term use by humans one might expect plants used in traditional medicine to have low toxicity (ELGORASHI *et al*., 2003). However, recent investigations have revealed that many plants used as food or in traditional medicine have mutagenic effects in *in vitro* assays (SCHIMMER *et al*., 1988, 1994; HIGASHIMOTO *et al*., 1993; KASSIE *et al*., 1996).

A mutagen is any agent (physical or environmental) that can induce a genetic mutation or can increase the rate of mutation. Chemicals that induce mutations can potentially damage the germ line and cause fertility problems and mutations in future generations. These chemicals are also capable of inducing cancer. This concern has driven most of the mutagenicity testing programs. Identification of substance capable of inducing mutations has become an important procedure in safety assessment (MORTELMANS and ZEIGER, 2000). Mutation events are involved in several degenerative diseases, such as cancer and arteriosclerosis (DE FLORA, 1998). VARANDA *et al*., (2002) reported many plants containing mutagenic compounds, such as furocoumarins, anthraquinones (DE SÁ FERREIRA and FERRÃO VARGAS, 1999) and flavonoids (RIETJENS *et al*., 2005).

Mutations can occur as gene (point) mutations, where only a single base is modified or (a relatively) few bases are inserted or deleted, as large deletions or

rearrangements of DNA, as chromosome break or rearrangements, or as gain or loss of whole chromosomes (MORTELMANS and ZEIGER, 2000). Gene mutations that cause a change in the growth requirements can easily be detected using bacteria. Chromosome damage in mammalian cells is typically measured by observing the chromosomes under magnification for breaks or rearrangements (MORTELMANS and ZEIGER, 2000).

The Ames test (*Salmonella typhimurium*/microsome assay) is used as a short-term bacterial assay for identifying substance that can produce genetic damage that lead to gene mutations. The Ames test is also used world-wide as an initial screen to determine the mutagenic potential of new chemicals and drugs (MORTELMANS and ZEIGER, 2000). This test uses several *Salmonella* strains with pre-existing mutations that leave the bacteria unable to synthesize the required amino acid histidine and therefore unable to grow and form colonies in its absence. New mutation of the pre-existing mutations can restore the gene function and allow the cells to synthesize histidine. Newly mutated cells can grow in the absence of histidine and form colonies.

Research has already shown that a lot of plants which are used in traditional medicine have *in vitro* mutagenic (CARDOSO *et al*., 2006; DÉCIGA-CAMPOS *et al*., 2007; MOHD-FUAT *et al*., 2007) or toxic and carcinogenic (DE SÁ FERREIRA and FERRÃO VARGAS, 1999) properties. Plants that show clear mutagenic properties should be considered as potentially unsafe and they require further testing before they can be recommended for use. Mutagenicity can also be useful as an anticancer tool, as most anticancer drugs are mutagenic for example the spindle-disturbing substances taxol and vinblastine (VERSCHAEVE and VAN STADEN, 2008). Mutagenic potential in plants can be considered interesting for therapeutic use and merit further in depth investigations of their pharmacological properties.

4.6.2. Materials and Methods

Mutagenicity was tested using the *Salmonella*/microsome assay without metabolic activation, based on the plate-incorporation method (MARON and AMES, 1983). Non-aqueous extracts were dissolved in 10% DMSO. For each sample, three dilutions 50, 500, 5000 µg/ml of the sample were used. *Salmonella typhimurium* strainTA98 was incubated in Oxoid Nutrient Broth No. 2 for 16 h at 37 ℃ in a water bath on an orbital shaker. A hundred microlitre of the test solutions (plant extract, solvent/negative and positive controls) were placed in a test-tube and 500 µl of phosphate buffer were added before the addition of 100 µl of overnight bacterial culture (2 x 10^8 cell/ml). Two millilitres of top agar containing histidine-biotin were added to this mixture. The mixture was then poured over the surface on a minimal agar plate and incubated (inverted in the dark) at 37 ℃ for 48 h. After incubation, revertant colonies were counted and compared to the number of revertant colonies in the solvent control. An extract was considered mutagenic when the mean number of revertants was at least double that of the solvent control. Each concentration per sample was tested three times in two different experiments. The negative control was distilled water and the positive control 4NQO (4-nitroquinoline-1-oxide).

4.6.2.1. Statistical analysis

Genotoxicity results were expressed as mean ± SE of three independent experiments and $P < 0.05$ was considered significant. The software used was the Graph Pad Prism Version 4.00 statistical software program for Windows (GraphPad Software Inc.).

4.6.3. Results and Discussion

The results of the Ames test to detect genetic damage, induced directly or indirectly, using *S. typhimurium* (TA98) are shown in Table 4.3. The results revealed that all the extracts were non-mutagenic towards *S. typhimurium* strain TA98. The average revertants observed ranged between 9.3 and 23.7 for all the extracts at all the concentrations whilst it was 22.0 for the negative control (water)

and 64.4 for the positive control (4NQO). An extract is considered mutagenic if the number of revertants per plate was at least double that of the spontaneous revertants (negative control) (BULMER *et al*., 2007).

Table 4.3: Number of His^+ revertants in *Salmonella typhimurium* strain TA98 produced by *A. natalensis* and *A. amatymbica* leaf and root extracts.

Sample	Extracting solvent	Number of His^+ revertants		
		50µg/ml	500 µg/ml	5000µg/ml
A. natalensis leaf	PE	23.7 ± 0.3	19.7 ± 1.8	23.7 ± 0.3
	DCM	19.0 ± 1.0	18.0 ± 4.7	19.0 ± 1.0
	EtOH	13.3 ± 2.0	12.3 ± 3.5	13.3 ± 2.0
A. natalensis rhizome	PE	22.0 ± 3.5	19.0 ± 2.0	22.0 ± 3.5
	DCM	20.3 ± 2.3	18.0 ± 1.0	20.3 ± 2.3
	EtOH	16.0 ± 2.5	15.0 ± 3.5	16.0 ± 2.5
A. amatymbica rhizome	PE	20.3 ± 2.8	23.0 ± 1.5	20.3 ± 2.8
	DCM	20.3 ± 2.3	18.0 ± 1.0	20.3 ± 2.3
	EtOH	13.0 ± 0.6	13.0 ± 2.6	13.0 ± 0.6
4NQO (+ve control, 2 µg/ml)			64.4 ± 0.9	
Water (-ve control)			22.0 ± 2.3	

Number of His^+ revertants/plate: mean values of three replicates, repeated three times.

+ve – positive.

-ve – negative.

Plants synthesize toxic substances, which in nature act as a defence against infections, insects and herbivores, but also may affect the organisms that feed on them. Hence an assessment of cytotoxic and mutagenic potential of extracts used in traditional medicine is necessary to ensure their safe use (CAVALCANTI *et al*., 2006). The results revealed that the extracts of the two species investigated were not mutagenic towards the tester strain (Table 4.3). The absence of a mutagenic response by plant extracts against *S. typhimurium* bacterial strains in the Ames assay is a positive step forward in determining the safe use of these plants in traditional medicine (REID *et al*., 2006). According to REID *et al*. (2006) plant

extracts exhibiting a positive response and a mutagenic effect on bacteria tester strain need to be extensively investigated to determine their possible mutagenicity towards humans as their safe use in traditional medicine is questionable.

4.7. Conclusions

A. natalensis, which is not as popular as *A. amatymbica* in traditional medicine, has both high antimicrobial and anti-inflammatory activity. The results revealed that *A. natalensis* leaves, which are normally used as a vegetable and not reported to be used in traditional medicine, has higher antifungal and anti-inflammatory activity compared to the rhizome of the same plant and that of *A. amatymbica*. Both *A. natalensis* and *A. amatymbica* did not yield genotoxic activity against *Salmonella typhimurium* TA98 which suggests that they are probably safe for medicinal use, though other tests need to be conducted to confirm its safety. *A. natalensis* can be used as a substitute in a conservation strategy for *A. amatymbica* which is highly threatened.

CHAPTER 5. GENERAL CONCLUSIONS

For many years medicinal plants have been used as a primary source of medicines to improve the health of people. It is now well documented that medicinal plants are over-utilised and this could lead to the extinction of many species. Recently there have been a number of attempts to cultivate some of the important medicinal plants (KULKARNI *et al.,* 2005 a, b; 2006; 2007; SPARG *et al*., 2005a). Propagation of medicinal plants is aimed at increasing the number of the plants and at the same time conserving them. Some *Alepidea* species are heavily traded and threatened medicinal plants in South Africa, and propagation of this plant by seed germination may be considered as a key tool in their conservation.

Favourable conditions necessary for seed germination of two *Alepidea* species (*A. amatymbica* and *A. natalensis*) were investigated. This was done by subjecting the seed to different treatments such as temperatures, light, sowing depth and storage conditions. Stimulation of seed germination was attempted using chemical regulators, smoke-water and butenolide.

The moisture content of *A. amatymbica* was 4% while that of *A. natalensis* was 7%. This suggests that the seeds can be stored for long period without fungal infections since their moisture contents are low. The seeds of both species achieved high germination percentages in a short period of time (MGT) under light conditions and at 25 ℃. This suggests that both species are positively photoblastic. After storing the seeds at 5 ℃ (cold stratification) for 14 days (*A. amatymbica*) and 28 days *(A. natalensis*), the percentage germination of both species was significantly increased. This confirms that these two species also exhibit morphophysiological dormancy (MPD) as is the case with other Apiaceae species (BASKIN and BASKIN, 1984). The two *Alepidea* species need to be sown near the soil surface for germination because they are light dependent. A sowing depth of 0.5 cm was the best. To improve germination of the two *Alepidea* species,

smoke-water and GA_3 where tested and they both significantly improved the germination of both species.

Traditional healers use some *Alepide*a species to treat different ailments including abdominal disorders, respiratory tract infections and colds. In this study different plant extracts of *A. amatymbica* (rhizomes) and *A. natalensis* (leaves and rhizomes) were screened for antimicrobial as well as anti-inflammatory properties to validate the reported medicinal properties of these species and also to test for possible genotoxic effects.

Results obtained from the antimicrobial bioassays showed different levels of activity against Gram-positive and Gram-negative bacteria as well as *Candida albicans* by the extracts. The water extracts of *A. natalensis* rhizomes exhibited high activity against the four bacterial strains tested (*Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus).* The *A. natalensis* leaf extracts exhibited the highest antifungal activity against *C. albicans*. The results validate the use of *A. amatymbica* extracts in traditional medicine. The activity observed in extracts of *A. natalensis* suggests that the species can be used as an alternative to *A. amatymbica*. The PE and DCM extracts of *A. amatymbica* and *A. natalensis* showed high activity against COX-1 and COX-2 enzymes. The weak activity observed from the other extracts does not necessarily imply the absence of bioactive agents. Genotoxicity results revealed that both *A. natalensis* and *A. amatymbica* could be used safely in traditional medicine.

This study has shown that the two *Alepidea* species can be propagated easily by via seed germination. The study also shows the importance of the two species as medicinal plants. It also revealed that *A. natalensis* can be used as a medicinal plant just as effectively as *A. amatymbica*.

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